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HEAD: PROFESSOR GÖSTA T. HULTQUIST

STUDIES ON AVIAN ERYTHROLEUKEMIA

2. *Host Responses after Different Virus Doses*

By

JAN PONTÉN[1]

Received 29 xii 60

The host response after a large dose of chicken erythroblastosis virus (CEV) has been investigated in considerable detail (9, 10). The response has a uniform character and can be divided into three fairly distinct phases (silent, medullary and extramedullary) according to the developmental stage of the disease (9).

It is well known that the survival time is lengthened when the virus dose in decreased and this relationship has been found useful for bio-assay purpose (4). The morphologic foundation for such an increase of the survival time has not been systematically investigated. It is for instance not known whether the increase is due solely to an increase of the silent phase (incubation period) or whether other mechanisms are also active.

This paper describes experiments where the development of erythro

... of animals inoculated with different amounts of CEV.

EXPERIMENTAL

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Fig 2

Series A Bone marrow section from animal dying 23 days after inoculation of erythroblastosis virus The peripheral blood presented pancytopenia and a few circulating erythroblasts ("anemic erythroleukemia") The section shows a moderately cellular bone marrow with a fairly large number of blastcells Fibrosis and accumulation of lymphocytes are also noted No or only very sparse myelo and thrombopoesis is seen Giemsa

the last week before death This animal had a high red cell count of 2 6 mill on the 7th day The red cells then rapidly decreased in number until the 7th day when a pronounced anemia had developed

The *thrombocytes* were essentially within normal limits except in the animal referred to above This bird showed a rather pronounced thrombopenia during the last week before death

The *white blood cell* counts were within normal limits except in the animal with thrombopenia and anemia which died on day 24 This bird had a marked leukopenia which involved mainly the granulocytes and during the last week counts around 8,000 W B C per cumm blood were obtained (normal 25,000)

At autopsy all birds had pronounced spleno- and hepatomegalia except the bird dying on the 24th day which had a spleen and a liver of normal size This bird was emaciated and had marked pallor of the skin and the organs

Histologic bone-marrow sections from the pancytopenic bird dying on day 24 revealed a diffusely involved moderately cellular marrow with very immature blast cells A conspicuous feature was an almost complete absence of myelopoietic and thrombopoietic foci A slight fibrosis was further noted (Fig 2)

The bird dying on the 24th day thus showed several atypical features viz absence of spleno- and hepatomegalia, profound pancytopenia and only a few circulating erythroblasts, a symptom complex which characterizes "anemic erythroleukemia' (6)

Series B 5 birds served as untreated controls All birds were punctured for blood cell counts on the same days

The erythrocytes (Fig 3) were essentially within normal limits ex-

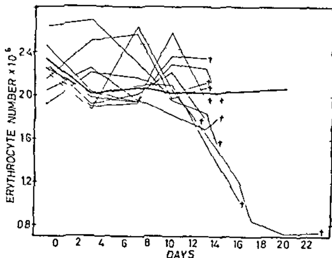


Fig 1

Series A Erythrocytes The thin uninterrupted lines indicate the birds inoculated with virus Heavy crosses indicate the days on which death occurred The heavy black line connects the means of a control group of 10 chickens The interrupted lines indicate the range of + 2 6 times the standard deviation

on May Grunewald Giemsa stained preparations As before (9) the erythroblasts were divided into three classes (I II III) according to the degree of maturity

The *virus isolations* in series F were made from bone marrow which was either dissected at autopsy or aspirated from the femur diaphysis *in vivo* After removal *the bone marrow was frozen and stored at —30° C for 1 to 3 weeks* When the bone marrow was tested for infective CFV a 1 per cent suspension was made up with phys saline homogenized and centrifuged at 3000 r p m The supernatant was inoculated intravenously into groups of ten chicks (age 10 days) which were then observed for 30 days All leukemic deaths were recorded Three different lots of chickens were used In order to ascertain that no great difference in sensitivity existed between the different lots of chickens a standard CFV solution was inoculated into ten chickens of each lot No significant difference in the response towards the standard CFV was found and it could be concluded that the three lots of test chickens used did not differ greatly from each other in sensitivity towards CFV infection

RESULTS

Series A 10 birds served as untreated controls Blood cell counts were made from these at the same time as from the animals inoculated with CLV

The erythrocytes (Fig 1) showed in several instances a *tendency to* hypernormal values during the first 10 days The values were however only significantly pathologic when they were compared to the control values of the respective days but not if they were compared to the original values of the control group The hypernormal values were therefore only relative and at least partly due to an absence of the anemizing effect which blood sampling *per se* caused among the controls

Blood was sampled with intervals of approximately 3 days This caused a slight initial fall of the erythrocyte values *among the controls*

One animal dying on the 24th day had a pronounced anemia during



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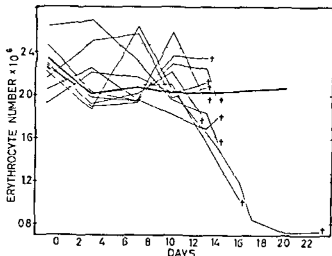


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One animal dying on the 24th day had a pronounced anemia during

The *differential red cell* counts revealed abnormalities in all birds except the survivor which had red cell counts within the normal range. The number of erythroblasts was moderate and the highest percentage of blast cells encountered in any bird of this series was 10 per cent. Evidence of a disturbed erythropoiesis was obtained in three survivors in the peripheral blood smears from day 25. Two showed circulating erythroblasts and the third had an increased number of circulating polychromatic erythrocytes. The highest percentage of erythroblasts in the red cell counts from the survivors was 0.6 per cent.

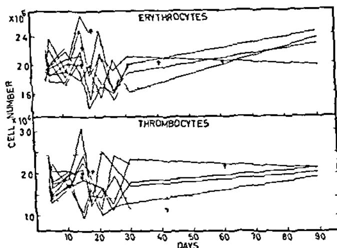


Fig. 4

Series C. Erythrocyte and thrombocyte counts. Same symbols as in Fig. 1.

The *bone marrow smears* (day 25) of three survivors showed an increased cellularity and hyperplastic erythropoiesis. Myelopoiesis was normal. The fourth survivor failed to show any definite abnormality.

The *white blood cell* counts were normal.

The *thrombocytes* (Fig. 4) *were outside the normal limits on several* occasions. One animal exhibited a rise on the 16th day, the other pathologic values were subnormal. The same three of the four survivors that were anemic had episodes of thrombocytopenia.

Series D. Cell counts were made from 10 control chickens at the same time as from the birds inoculated with CEA.

The *erythrocytes* (Fig. 5) *tended to be subnormal after the sixth day* and most values were below the mean of the control group and a few values were below the limit of 2.6 times the standard deviation of the control group mean. Two animals which died on the 11th and 20th day had pronounced terminal anemia. Both survivors showed abnormal values, one a slightly hypernormal value and the other a moderate anemia around the 28th day.

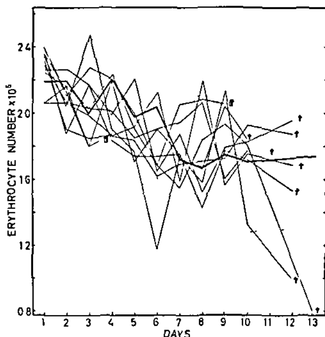


Fig 3

Series B Erythrocyte counts The thin uninterrupted lines indicate the birds inoculated with virus Heavy crosses with a frame indicate intercurrent deaths unrelated to leukemia The heavy black line connects the means of a control group of 5 animals The interrupted lines indicate the range of ± 2 6 times the standard deviation

cept in two animals dying on day 12 and 13 with subnormal values One of these had an anemic episode on the sixth day Previous observations (9) of the anemizing effect of frequent blood sampling *per se* were confirmed as seen from the decrease of the erythrocyte counts that took place among the controls

The *thrombocytes* were within the normal range except the terminal value of the bird dying on the 13th day which was slightly subnormal (*13,000 per cumm*)

The *white blood cell* counts were normal except two slightly subnormal values on days 12 and 13

Series C To obtain control values cell counts were made from 10 normal birds on day 0, 50 and 100

The *erythrocytes* (Fig 4) showed episodes of both subnormal and hypernormal values between the 15th and 30th day One bird died on the 20th day with a slightly hypernormal value Three of the four survivors had on one or more occasions subnormal red cell counts between the 15th and 30th day The fourth survivor had no definitely abnormal value (one red cell count at the upper normal limit on day 18)

It was further observed that the normal values for erythrocytes and thrombocytes were somewhat lower in the Edo White Leghorn strain used in series C than in the Borje White Leghorn strain used in series A and B

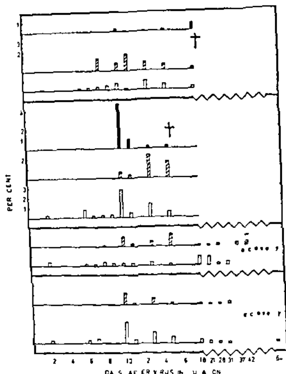


Fig 7

Series D Four examples of differential red cell counts obtained from peripheral blood smears Unfilled columns indicate polychromatic erythrocytes hatched columns indicate erythroblast of type II and III and black columns indicate erythroblasts of type I The two crosses indicate the days on which the animals died from leukemia

bocytosis, but eventually returned to normal values No thrombocytopenia was noted The thrombocytes were unaffected by the blood sampling *per se*

Four examples of the series of *differential red cell counts* carried out are given in Figure 7 It is seen that the two recovering birds were abnormal during long periods

The bird illustrated lowermost showed a pronounced thrombocytosis (Fig 6) until at least the 31st day and had a slight elevation of the red cell number on the 31st day Already in the smears from the 2nd, 6th and 7th day polychromatic erythrocytes were met with The percentage was however not above the limit of 0 5 and thus not significantly raised (9) On the 10th day significantly increased numbers of polychromatic erythrocytes were noted and the earliest erythroblasts of type II and III appeared The number of erythroblasts slowly fell but as late as on the 31st day 0 3 per cent were observed After this day the red cells showed no abnormality on the three further occasions when smears were made

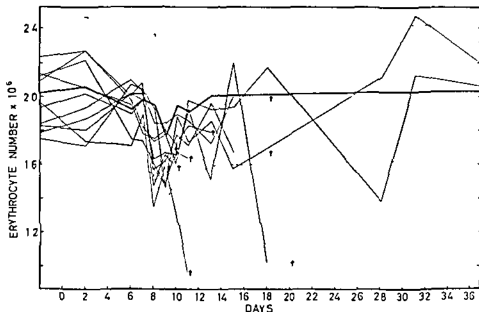


Fig 5
Series D Erythrocyte counts Same symbols as in Fig 1.

The control group kept a normal red cell level except around the 9th day when a series of daily blood samples were performed which caused a transient slight anemia

The *thrombocytes* (Fig 6) showed a slight increasing tendency in some animals around the 6th day. After the 13th day four birds showed marked thrombocytosis One of these had a hypernormal value 3 days before death and an other returned to normal before death The two surviving birds had long periods of 2–3 weeks with pronounced throm-

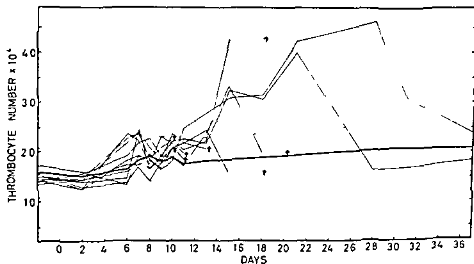


Fig 6
Series D Thrombocytes counts Same symbols as in Fig 1

The other bird which recovered had erythroblasts of type II and III circulating during a period of approximately one month beginning on the 8th day and on a single occasion (day 42) a few erythroblasts of type I were also noted Concomitantly with the decrease of erythroblasts around the 20th day a transient increase of polychromatic erythrocytes was observed

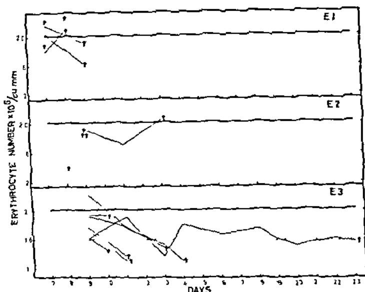


Fig 8

Series F *Erythrocyte counts* Same symbols as in Fig 1

The two birds which recovered were observed 3 months and hematologic control which included bone marrow aspiration revealed nothing abnormal at the end of this period

The two chickens whose differential erythrocyte counts are illustrated in the upper half of Fig 7 exemplify animals dying from leukemia In one animal (the diagram next to the top) polychromatic erythrocytes were detected early and on the sixth day a significant rise to 1 per cent was observed On the 10th day a burst of erythroblasts of type I was detected These cells were then gradually replaced by more mature erythroblasts The polychromatic erythrocytes never disappeared from the blood

In the bird of the upper compartment of Figure 7 a somewhat different sequence of events was observed Polychromatic erythrocytes increased slowly until the 13th day after which they slowly declined Erythroblasts of type II and III suddenly appeared in the smear of the 8th day After a period of around one week they declined to reach the low value of 0 3 per cent on the last occasion when a smear was made

TABLE 1

Basical Data for the Experiments

exp no	chick strain	chick age (day 0)*	no of animals §	virus dilution factor	survivors (per cent)	median survival time (days)	K-value †	month when exp was performed	virus isolation
A	W L (Borje)	20	10		0	13.5	7.0	june	not performed
B		27	10		0	12	8.5	june	–
C	W L (I do)	19	9		44	60	2.0	july	–
D		16	10	–	20	13	6.2	oct	–
E 1		10	6	1	0	8	12.9	nov	–
E 2		10	9	10	0	9	10.9	nov	
E 3		10	10	10^3	0	10.5	8.8	nov	–
F 1		25	14	1	10	10	8.8	april	performed
F 2		25	15	10	34	12	6.2	april	–
F 3		25	29	10^2	9	11.5	6.9	april	not performed
F 4		25	68	10^3	33	16	4.7	april	performed
F 5		25	13	10^4	42	41	3.1	april	–

* day 0 = day when experiment was started through intravenous inoculation of CLV

§ corrected for intercurrent deaths

† The arithmetic mean for the reciprocals of the survival time (including the survivors, which were regarded as having an infinite survival time) See ref 8

occurrence of large number of polychromatic erythrocytes At the same time (Fig 8, E 3) the red cell count rose On day 10 an exacerbation was noted with erythroblasts of type II and III circulating in moderate numbers until the animal was killed on day 24

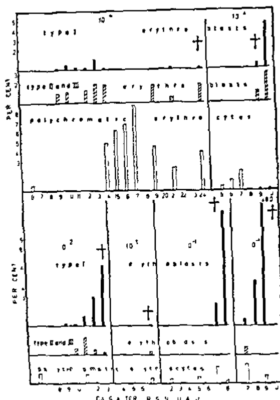


Fig 9

Series F Six examples of differential red cell counts obtained from peripheral blood smears Same symbols as in Fig 7

Bone marrow smears The two cases dying on day 7 showed intense involvement of the bone marrow (Fig 10), which was filled with erythroblasts of a very immature type In the acute cases dying between day 8 and 12 a similar picture was seen with an abundance of immature erythroblasts of type I The animals dying on days 13 and 14 presented a more mature appearance at death with a preponderance of more differentiated erythroblasts of type II

The animal of the group inoculated with dilution 10^{-4} which lived 23 days had on day 20 a moderately cellular smear, which showed evidence of maturation into erythrocytes Terminally the bone marrow smear was of essentially the same appearance (Fig 11) and still no complete maturation block could be discerned

Erythroblasts of type I appeared later (day 10) and exhibited a terminal rise

The *white blood cell counts* were normal throughout

Series E In this series the terminal events were most closely studied and preceding interference by making blood cell counts was avoided as much as possible No control animals were included, the normal values given were calculated from untreated birds of the same age as the chickens inoculated with CEV Since death due to erythroleukemia is only preceded by a short period during which the animals show clinical signs of disease (weakness, lassitude, loss of appetite and ruffling up of feathers), several animals were lost before any cell counts could be done

The *erythrocytes* (Fig 8) showed no anemia with dilution 10^{-1} (E 1) and only one anemic value was encountered with dilution 10^{-2} (E 2) In the dose-group inoculated with dilution 10^{-4} (E 3) six of nine animals were anemic at death

The *thrombocytes* showed one slightly hypernormal value in each group In one animal (dilution 10^{-4}), killed on the 23rd day, a series of low values, one of which (day 19) was significantly below the normal limit, were observed during the last week before death

Representative examples of the *differential red cell counts* are given in Fig 9

Two of the three birds of Fig 9 inoculated with the highest virus dose (survival time 8 and 9 days) showed the response typical for acute cases of erythroleukemia (9) Initially there was an increase of polychromatic erythrocytes The leukemic phase was begun on day 7 (or 6) when erythroblasts of type I entered the blood stream in large amounts They were exclusively of type I in one animal In the other animal 1 per cent erythroblasts of type II and III was observed on day 7 but on day 8 and 9 they had wholly disappeared Terminally the polychromatic erythrocytes were absent or very few in the two cases described

The third animal inoculated with dilution 10^{-1} and illustrated in Fig 9 died on the 7th day It exemplifies certain peracute cases which may die before changes in the peripheral blood have had time to develop

The birds of Figure 9 inoculated with virus dose 10^{-2} showed the usual response in acute leukemia The bird to the left in the lower row had clearly detectable "waves" of erythrocyte precursors of increasing immaturity and terminated with an almost total dominance by large very immature erythroblasts of type I The other (to the right in the upper row of the figure) had a transient increase of polychromatic erythrocytes and large numbers of erythroblasts of all types at death

The bird illustrated in the upper left corner of Figure 9 showed a response sometimes seen in "chronic" cases initiated with a low virus dose Already on the sixth day, when the first smear was made, a slight increase of circulating polychromatic erythrocytes was noted After day 14 a remission occurred with a disappearance of the erythroblasts and

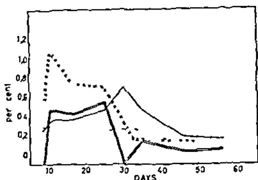


Fig 12

Series F Percentage of different types of erythroblasts in peripheral blood smears

= = = = -	F1 F4	-	I
— — — —	F5	-	I
———	F5		II and III
═══ dose group	F1 F4	erythroblasts type	II and III

Series F In this series the differential red cell counts in the peripheral blood were particularly studied During the development of the disease 681 blood smears were made from the 152 chickens included in the experiment In the figures the values from the four dose groups (F1–F4) have been combined and compared to the values in dose group F 5 (where the inoculum contained the smallest amount of CLA) In the calculations the relatively mature erythroblasts of type II and III have been combined and compared with the immature erythroblasts of type I In Fig 12 the mean percentage of erythroblasts of different degree of maturity are depicted It is seen that in dose group F 5 there was on the average, a preponderance of differentiated erythroblasts at

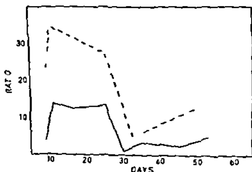


Fig 13

Series F Ratio erythroblasts type I erythroblasts type II and III (= Immaturity Ratio)
Uninterrupted line = dose group F5
Interrupted line = dose group F1 F4

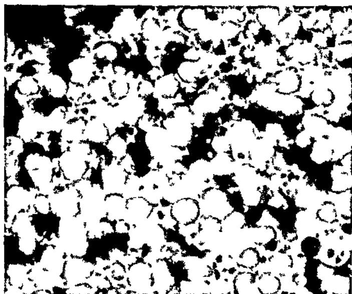


Fig 10

Series F 1 Virus dilution 10^{-1} Bone marrow smear in the terminal stage of a per acute case The bird died 7 days after erythroblastosis virus inoculation Only few erythroblasts were found in the peripheral blood The bone marrow was cellular and dominated by immature erythroblasts Maturation arrest as evidenced by the absence of maturing intermediates between immature erythroblasts and mature erythrocytes MGG

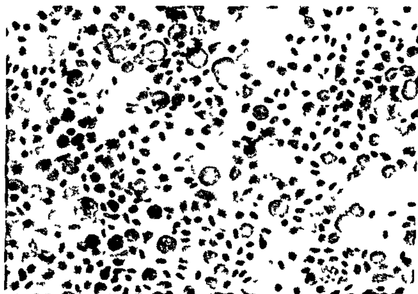


Fig 11

Series F 3 Virus dilution 10^{-4} Bone marrow smear taken on the 24th day from the animal depicted in Fig 9 Erythroblasts of all degrees of maturity indicating some differentiation in the red cell series A few myelocytes containing granules are also seen MGG

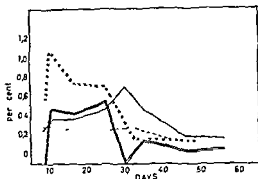


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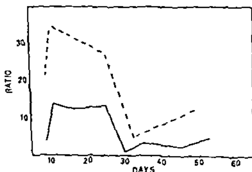


Fig 13

Series F Ratio erythroblasts type I/erythroblasts type II and III (= Immaturity Ratio)
Uninterrupted line = dose group F5
Interrupted line = dose group F1–F4

TABLE

Summary of the Results of CEV Isolations from the Bone Marrow Series F The Animal Infinite

Chick no	Group Relative CEV dose	9–12	13–15	16–18	19–21	22–24	DAYS 25–27	28–30	31–33	34–36
393	F5 10^{-4}	O		M	—		I	—		M —
404		M			+		M	+		M +
401		M	I	M	—	†				
457	F4 10^{-3}	M	M	I			M		O —	M +
449			M	O —				O	M	
463		M +	killed day 11							
508			M	M			M	O —		M
512		M	M	M				M +		M
514		I	M +	†						
516		M		I	O		M	M +		
529		M	M	I +			M		M +	
478	F2 10^{-1}	I +	†							
482		I +	†							
483		I +	†							
383	F1 1	M	M —	O			O	O +		
384		M	M	M +			I	M		O
388		M	I	M +			M	M —		
392		I +	†							

I = immaturity ratio > 5 (see text) i e immature type of differential red cell count in the peripheral blood
M = immaturity ratio ≤ 5 (see text) i e mature type of differential red cell count in the peripheral blood

all points of time except around day 10 and at the end of the experiment when the percentage of erythroblasts of type I were about equal to the percentage of type II and III Around day 30 the values differed appreciably and the differentiated erythroblasts showed a peak The curves for dose groups F1–F4 show that these animals which were inoculated with higher amounts of CEV, responded with more immature erythro-

2
Are Selecte l an l most Isolations Have Been Attempted from Animals with Prolonged or Survival Times

37 39	40 42	43 45	46 48	49 51	52 54	55 57	General character of erythroleukemia
+							Mature Blastcells (type I) around day 25 Died day 36
							Mature Survived
							Immature changing to mature Died day 21
+							Mature Blastcells (type I) around day 13 16 Died day 36
			M —				Mature Survived
							Indeterminate Killed day 11
		M —		I —	M	O	Mature Blastcells (type I) around day 48 Survived
I	M			M	O —		Mature Blastcells (type I) around day 36 Survived
							Immature changing to mature Died day 15
†							Mature Blastcells (type I) around day 16 Died day 34
M	M +	M				O +	Mature Blastcells (type I) Survived
							Immature Died day 9
							Immature Died day 9
							Immature Died day 9
M		M		M			Mature Survived
			O —	I		O +	Mature Blastcells (type I) around days 25 a 47 Survived
M		O +		I +	+		Mature changing to immature Died day 48
							Immature Died day 11

O = normal red cell differential count in the peripheral blood
\+ = pos t ve CFV isolation
— = negative CFV isclat on
† = death of animal due to leukemia

blasts and at all points of time, type I cells outnumbered the more differentiated erythroblasts

ratio erythroblasts
I . . following this ratio,
will be referred to as
the I R (= Immaturity Ratio) *From Fig* 13 can be seen that the I R

TABLE

Summary of the Results of CEV Isolations from the Bone Marrow Series I. The Animals Infected

Chick No.	Crit. Relative CEV dose	9-12	13-15	16-18	19-21	22-24	DAYS 25-27	28-30	31-33	34-36
393	1.5 · 10 4	O		M	—		I	—		M +
404		M			+		M	+		M +
401		M	I	M	—	†				
457	1.4 · 10 3	M	M	I			M		O —	M +
443			M	O —				O	M	
403		M +	killed day 11							
08			M	M			M	O —		M
1		M	M	M				M +		M
14		I	M +	†						
16		M		I	O		M	M +		
20		M	M	I +			M		M +	
478	1.2 · 10 1	I +	†							
482		I +	†							
483		I +	†							
383	1.1	M	M —	O			O	O +		
384	1	M	M	M +			I	M		O
388		M	I	M +			M	M —		
392		I +	†							

I = immaturity ratio > 5 (see text) i.e. immature type of differential red cell count in the peripheral blood

M = immaturity ratio ≤ 5 (see text) i.e. mature type of differential red cell count in the peripheral blood

all points of time except around day 10 and at the end of the experiment when the percentage of erythroblasts of type I were about equal to the percentage of type II and III. Around day 30 the values differed appreciably and the differentiated erythroblasts showed a peak. The curves for dose groups F 1-F 4 show that those animals which were inoculated with higher amounts of CEV responded with more immature erythro

TABLE 3

Summary of the Results. The Dose Groups Have Been Arranged According to the A2 Values which Give a Rough Estimate of the Infective CFU Content of the Inoculates

	Red cells				Thrombocytes		Granulopenia	Presence of F. in erythroleukemia	Survivors %	Signs of erythroleukemia	A2
	hypernormal values	terminal anemia	anemic episodes	deg. of red c. maturity	hypernormal values	thrombocytopenia					
I 1	n.i	0%	0%	immature	n.i	0%	n.i	0%	0	100%	12.9
I 2	n.i	20%	0%	immature	n.i	0%	n.i	0%	0	100%	10.9
I 3	n.i	50%	10%	10% mature	n.i	10%	n.i	0%	0	100%	8.8
I 1	0	60%	10%	10% mature	n.i	n.i	n.i	0%	10	100%	8.8
B	0	20%	0%	n.i	0%	10%	20%	0%	0	100%	8.5
A	40%	20%	0%	gen. immature	0%	10%	10%	10%	0	100%	7.0
I 3	0	88%	n.i	20% mature	n.i	n.i	n.i	n.i	9	100%	6.9
D	10%	20%	50%	gen. mature	40%	0%	0%	0%	20	100%	6.2
I 2	n.i	90%	40%	immature & mature	n.i	n.i	n.i	n.i	34	100%	6.2
I 4	n.i	80%	n.i	immature & mature	n.i	n.i	n.i	n.i	33	100%	4.7
I 5	n.i	0%	40%	gen. mature	n.i	n.i	n.i	n.i	42	100%	3.1
C	10%	0%	88%	gen. mature	11%	88%	0%	0%	44	88%	2.0

n.i. = not investigated

was consistently higher in dose groups F 1–F 4 than in dose group F 5 This difference was most pronounced between day 12 and day 25

In Figures 12 and 13 mean values have been calculated Such values do not however indicate the great individual difference that existed These differences increased with a decreasing CEV dose so that, in dose group F 1, the animals, in general, responded in a uniform manner, whereas in dose group F 5 great individual differences existed A few animals responded with acute, immature leukemias of the type met with in dose groups inoculated with high amounts of CEV The majority, however, had leukemias characterized by rather mature cells with obvious signs of differentiation both in the bone marrow and the peripheral blood The "chronic" cases pursued one of the following three types of clinical course 1) After one or more incomplete remissions followed by more or less acute exacerbations, all signs of leukemia disappeared and a complete remission, at least within the limited observation period of 3 months, followed 2) After one or more exacerbations the leukemia stabilized itself af a "chronic" type of leukemia from which the animal eventually succumbed 3) After a "chronic" course an "acute", immature and lethal exacerbation ensued which rapidly killed the bird

Virus isolations Table 2 summarizes the results of virus isolations from bone marrow extracts As seen from the table all tested birds dying before day 13 (No 478, 482, 483 and 392) were positive for CEV They were all more or less typical "acute" cases with I R > 5

Birds dying later than day 12 showed more variable clinical pictures Several presented a mature "chronic" type of leukemia with a low I R (No 393, 401, 457, 514, 516) Only one of these was negative (No 401), the others had detectable amounts of CEV in the bone marrow at their death The bird (No 388) dying on day 48 with an immature leukemia was positive for CEV Thus, with only one exception, the birds which died from leukemia, were positive for CEV irrespective of the initiating dose of CEV and irrespective of the terminal blood picture In three of these birds CEV isolations were attempted on more than one occasion In two animals (No 393 and 457) three attempts to isolate CEV at various points of time after infection were negative In the third animal (No 388) CEV was recovered on two occasions (day 16 and day 43) in addition to the recovery of CEV on the day the bird was sacrificed

All surviving birds included in Table 2 showed evidence of a disturbance in the red cell series in the form of circulating erythroblasts From five of the seven survivors CEV was isolated on one or more occasions No definite correlation could be revealed between the peripheral red cell picture and the presence of CEV in the survivors Thus, in birds No 529 and 404, CEV was detected on six occasions when the blood picture was either normal or showed only a moderate number of erythroblasts of type II and III Birds No 449 and 508 on the other hand, were negative on all five occasions tested in spite of a similar blood picture

TABLE 3

Summary of the Results. The Dose Groups Have Been Arranged According to the V_2 Values which Give a Rough Estimate of the Infective CEV Content of the Inoculates

	Red cells				Thrombocytes		Granulopenia	Presence of FL in cytopenic erythroleukemia	Survivors %	Signs of erythroleukemia	V_2
	hyper-normal values	terminal anemia	anemic episodes	degree of maturity	hyper-normal values	thrombocytopenia					
I 1	n i	0%	0%	immature	n i	0%	n i	0%	0	100%	12.9
F 2	n i	20%	0%	immature	n i	0%	n i	0%	0	100%	10.9
I 3	n i	50%	10%	10% mature	n i	10%	n i	0%	0	100%	8.8
I 1	0	60%	10%	10% mature	n i	n i	n i	0%	10	100%	8.8
B	0	20%	0%	n i	0%	10%	20%	0%	0	100%	8.5
A	40%	20%	0%	gen. immature	0%	10%	10%	10%	0	100%	7.0
F 3	0	88%	n i	20% mature	n i	n i	n i	n i	9	100%	6.9
D	10%	20%	50%	gen. mature	40%	0%	0%	0%	20	100%	6.2
I 2	n i	90%	40%	immature & mature	n i	n i	n i	n i	34	100%	6.2
I 4	n i	80%	n i	immature & mature	n i	n i	n i	n i	33	100%	4.7
I 5	n i	0%	40%	gen. mature	n i	n i	n i	n i	42	100%	3.1
C	10%	0%	88%	gen. mature	11%	88%	0%	0%	44	88%	2.0

n i = not investigated

DISCUSSION

To facilitate a comparison between the different experiments and to illustrate the differences which were found between the different dose groups, Table 3 has been made In this table the dose groups have been arranged according to their A_c-values (see Table 1) which give a rough estimate of the potency of the CEV inocula

Before commenting on the table it seems appropriate to point out that different lots of chickens with probably a different degree of "natural" resistance against infection with CEV have been used and that therefore the correlations that exist between the initiating dose and the chicken response are approximate Nevertheless the table seems to establish the following facts

Reversible erythroleukemia is not uncommon and increases in frequency with a decreasing dose of CEV *Engelbreth Holm* (cf 6) noticed complete remissions in adult fowl but expressed the opinion that young chickens always succumbed to infection In the present experiments a large number of complete remissions were noted in young chickens This may be due to the thoroughness by which the animals were observed or to differences in the chicken strains used In series F 5 all birds showed evidence of infection, but over 40 per cent of the animals recovered The findings closely parallel the findings with Rous sarcoma virus, where regression of tumours is often observed (3, 13)

With a decreasing CEV dose the leukemias had a more variable course characterized by remissions and exacerbations during an extended period of time This is attested by the number of anemic episodes which increased with a decreasing CEV dose The number of animals exhibiting terminal anemia increased with a decreasing CEV dose, with the exception of the dose groups (F 5 and C) apparently containing the lowest amounts of CEV, where no cases with a terminal anemia were observed

Leukocyte counts were made in series A–D In general no great abnormalities were detected The animal with "anemic erythroleukemia" in series A was an exception and showed a pronounced leukopenia

The thrombocytes were in contrast often abnormal and both pathologically low and high counts were observed The thrombocytes were not systematically counted and definite conclusions are therefore difficult to arrive at It seemed however as if detectable thrombocytopoietic disturbances were especially common after low CEV doses A close relationship between erythro- and thrombocytopoiesis has been claimed for chickens (14) and in the light of this fact it is not implausible that CEV not only affects erythropoietic cells specifically but also may cause specific disturbances in thrombocytopoiesis

The main purpose of the present work was to find out whether the increase of the survival time which is observed after decreasing the initiating CEV dose was solely due to an increase of the incubation (induction) time *i e* the period during which no morphologic signs of

disease are apparent It is obvious that this is not the case Instead there was only a moderate increase of the time period which passed before the first abnormal red cells entered the blood If the bone marrow had been investigated systematically it is not improbable that this increase would have been even smaller or perhaps not at all apparent because erythroblast proliferation is known to precede the entrance of immature cells into the circulation by a definite interval (4 9 10 12) The lenghtening of the survival time was mainly due to a prolongation of the period during which signs of a disturbed erythropoiesis in the form of circulating erythroblasts could be revealed After low CEV doses erythroblasts could be found circulating during several weeks This is in marked contrast to the few days passing between the entrance of the first erythroblasts into the blood and the death of the animal after a massive CEV dose

Another difference was that erythroblasts of all degrees of maturity were often encountered after a small virus dose whereas a high dose nearly always resulted in the almost exclusive appearance of very immature blastcells After a high dose the resulting disease was rapidly fatal after a uniformly progressive course In contrast the leukemias induced by smaller CEV doses showed remissions and exacerbations and some of them even ended in apparent cures These observations probably mean that the chicken organism possesses defense mechanisms against erythroleukemias induced by inoculation of CEV

The most plausible explanation of the differences noted between the differentiated leukemias after low CEV doses and the undifferentiated after high doses is that this difference is only apparent and due to the number of "leukemic" foci existing in the bone marrow It is then assumed that the entrance of CEV into susceptible stem-cells results in a transformation of the cells into blast cells unable to differentiate further In the case of infection with a large CEV dose a large number of foci of infected blast cells are established Through the proliferation of these foci the normal remnants of bone marrow are overgrown and undifferentiated erythroblasts will rapidly dominate the scene After a small CEV dose only a few foci are primarily established These foci enlarge but during this process the part of the bone marrow which has not been infected adapts itself to the proliferating infected blast cells and reacts with a compensatory mechanism which expres[illegible] itself [illegible] of ery[illegible] times [illegible] after [illegible] [illegible] with an undifferentiated picture identical with what is seen in the "acute" cases after a high CEV dose

The purely morphological investigations reported here cannot however exclude another possible explanation of the difference noted between the immature types of erythroleukemia noted after large CEV doses and the mature types noted after smaller virus doses According

to this explanation a difference might exist on the cellular level If the susceptible cells are infected with a high virus:cell ratio the blast cells are completely prevented to undergo any maturation If the susceptible cells are infected with a low virus.cell ratio the infected cells are still able to mature to a greater or lesser extent The erythroblasts of mature types and perhaps even the polychromatic erythrocytes encountered in many chickens inoculated with small amounts of CEV may then be looked upon as beeing derived from "leukemic" blast cells and not from nonspecifically altered normal erythroblasts According to this view clones of erythroblasts carrying different amounts of virus (or different numbers of virus producing centres) determined by the amount of virus that originally reached the cells may coexist The clones carrying the largest numbers of virus producing centres per cell are unable to differentiate whereas the clones containing cells with smaller numbers of virus producing centres are still able to mature With the related Rous sarcoma virus (RSV) there are indications that a tumour cell population contains cells with very different amounts of virus (1, 5, 8, 11) If the degree of collagenisation can be taken as an indication of the degree of fibroblast maturity, the observation (cf 2) that fibrous *(and more slowly growing) Rous sarcomas are induced by small doses* of RSV might be a parallel to the results reported here

The virus isolations reported here have only been qualitative and they do not tell anything of the amounts of CEV existing in the bone marrow The biological method using young chickens as test objects is certainly not sensitive enough to detect small amounts of CEV Nevertheless the results are not inconsistent with the last mentioned explanation of the morphological findings, since in general the extracts from the bone marrow of the chickens with immature erythroleukemias were positive whereas several extracts from animals with mature leukemias failed to reveal the presence of detectable amounts of erythroblastosis virus

SUMMARY

The hematological response in chickens inoculated with different amounts of chicken erythroleukemia virus has been investigated

After a high virus dose the resulting leukemias were characterized by a rapid uniform course ending with a bone marrow and peripheral blood picture where very immature cells were the dominating cells whereas signs of differentiation in the red cell series were almost absent Anemia or thrombocytopenia were only seldom seen

After a low virus dose the resulting leukemias showed great individual differences Several cases had a remitting course and many ended in apparent cures Many animals presented a differentiated red cell picture with red cell precursors of all degrees of maturity even terminally Terminal anemia (except after very low virus doses) and thrombocytopenia were often seen In this way certain cases came to

resemble chronic leukemias rather than the acute leukemias usually seen

Thrombocytopoiesis was often disturbed possibly due to a specific virus effect not only on erythropoietic but also on thrombopoietic cells

Virus isolations were performed from the bone marrow from selected animals Chickens dying early and inoculated with higher amounts of erythroleukemia virus tended to be positive more often than animals with slowly developing chronic erythroleukemias Exceptions were however noted and animals were observed where virus was isolated in spite of normal hematological values

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4 [illegible] erythroblastosis I Titra 1120 1956

5 [illegible] —Huhnersarkom Neoplas

6

7

8

9 *Fonté* [illegible]

10 *Fonte* [illegible]

11 *Fr ne* [illegible]

12 *Ratel* [illegible]

13 *Rous* [illegible]

14 *Sug b* [illegible]

Embry 1 18 121 147 1926 [illegible] Publ Contr bs

THE EFFECT OF FIVE ULCERATING DOSES OF ALKYLDIMETHYLBENZYLAMMONIUMCHLORIDE (ZEPHIRAN) ON THE TUMOR PROMOTING ACTION OF POLYOXYETHYLENE SORBITAN MONOSTEARATE (TWEEN 60)

An Analysis of the Dermal Condition in Tumor Promotion

By

KAI DAMMERT[1]

Received 14 1 61

The role of fibrosis in promotion of skin tumors of mice was studied by *Salaman & Glendenning* in 1957 (17) The change was produced by some sclerosing agents such as phenol, but the authors did not arrive at any conclusive answer regarding its relationship to tumorigenesis

In an earlier study (*Dammert* 1957) it was shown that the promoting effect of many non ionic surface active agents was related to the ability of the agents to cause long lasting hyperplasia of the epidermis and thickening and inflammation of the dermis, yet without appreciable regressive changes of the latter It was demonstrated too, that the condition of fibrosis associated with degenerative changes of the dermal connective tissue as seen after repeated use of the cationic surface active agent alkyldimethylbenzylammoniumchloride (Zephiran) was inconsistent with favorable promotion This paper will report experiments, where trials was made to change the condition of the dermis with a period of treatment known to be deeply damaging before commencement of an effective promoting procedure

MATERIAL AND METHODS

Female Swiss albino mice bred at random in this laboratory were used They were two months old and weighed 22 25 g at the beginning of the experiments They were kept in plastic cages on wood shavings and fed Rockland diet in pellets and [illegible] per back was shawed with an electric [illegible] micals Only mice at resting phase were used The *painted area* was [illegible]

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DMBA was dissolved in acetone of reagent grade distilled before use, Zephiran was dissolved in distilled water Tween 60 was applied undiluted at 36 C DMBA was applied with a calibrated micropipette, Zephiran and Tween 60 with a regular glass dropper The mice were examined twice a week Every tumor induced was recorded on special diagrams and recharted by every examination with consideration of any changes in number, size, type or position of the tumors In the final results tumors in dead animals were included provided that they had lasted for at least 4 weeks before the death of the animal The latent period was calculated as a mean of the time of appearance of the first tumor in every tumor bearing animal Histological examination of all tumors in dead or killed animals was carried out

EXPERIMENTAL AND RESULTS

3 groups of animals 30 in each were used in tumor production experiments All 3 were initiated with 20 μg DMBA in acetone After one week, a period of 2 weeks followed during which 2 of the groups were treated with 5 applications of 6 4 per cent Zephiran Two drops of the watery solution containing 2 4 mg Zephiran was given 8, 12, 14 16 and 23 days after the initiation These animals were then left without treatment for an additional 3 weeks The third group did not receive any other treatment before the commencement of the period of the final treatment

6 weeks after the initiation the final treatment was started in all three groups Of the two groups receiving the intermediate treatment with Zephiran one was treated with one drop of Tween 60 daily, the other with one drop of 3 2 per cent watery solution (0 6 mg) of Zephiran thrice a week and the third lacking the inter-[illegible]

[illegible] the beginning of the final treatments and pairs of animals of the three other groups were killed at each time after 1, 2 4, 10 and 20 weeks of the respective final treatments

The Changes Caused by Zephiran Treatments prior to the Final Treatments

4 days after the first treatment the treated skin was slightly red and desquamating The animals were nervous and lively apparently because of tenderness of the treated back There was alternating erosions and [illegible]

[illegible] was heavily inflammed and thickened (Fig 2) The additional fourth application given at the 16th day still increased the deep, massive inflammation Thick crusts or greasy squamas covered the treated skin without signs of hair growth During the following week no new Zephiran treatments was given and the skin began to heal with detachment of the crusts In the end of this week the treated skin still was thicker than usual and some animals still had crusts, but most of them showed smooth epilated patches and scars in places of the former crusts The fur of the treated area of many animals had a "moth eaten" appearance The 5th treatment with 6 4 per cent Zephiran at the 23rd day did not any more affect the skin like the first treatment did Only a slight reactivation of the inflammatory redness and increase of thick-

THE EFFECT OF FIVE ULCERATING DOSES OF ALKYLDIMETHYLBENZYLAMMONIUMCHLORIDE (ZEPHIRAN) ON THE TUMOR PROMOTING ACTION OF POLYOXYETHYLENE SORBITAN MONOSTEARATE (TWEEN 60)

An Analysis of the Dermal Condition in Tumor Promotion

By

KAI DAMMERT[1]

Received 14.1.61

The role of fibrosis in promotion of skin tumors of mice was studied by *Salaman & Glendenning* in 1957 (17). The change was produced by some sclerosing agents such as phenol, but the authors did not arrive at any conclusive answer regarding its relationship to tumorigenesis.

In an earlier study (*Dammert* 1957) it was shown that the promoting effect of many non ionic surface active agents was related to the ability of the agents to cause long lasting hyperplasia of the epidermis and thickening and inflammation of the dermis, yet without appreciable regressive changes of the latter. It was demonstrated too, that the condition of fibrosis associated with degenerative changes of the dermal connective tissue as seen after repeated use of the cationic surface active agent alkyldimethylbenzylammoniumchloride (Zephiran) was inconsistent with favorable promotion. This paper will report experiments, where trials was made to change the condition of the dermis with a period of treatment known to be deeply damaging before commencement of an effective promoting procedure.

MATERIAL AND METHODS

Female Swiss albino mice bred at random in this laboratory were used. They were two months old and weighed 22–25 g at the beginning of the experiments. They were kept in plastic cages on wood shavings and fed Rockland diet in pellets and water ad libitum. An area of 1.5 × 1.5 cm on upper back was shaved with an electric clipper one week before the application of the chemicals. Only mice at resting phase of hair growth at the beginning of the treatment were used. The painted area was kept shorn with electric clippers or scissors. The chemicals used were 9,10 dimethyl 1,2 benzanthracene (DMBA) (Eastman Organic Chemicals) purified by chromatography, alkyldimethylbenzylammoniumchloride (Zephiran) (Winthrop Products Ltd.) and polyoxyethylene sorbitan monostearate (Tween 60) (Atlas Powder Co.)

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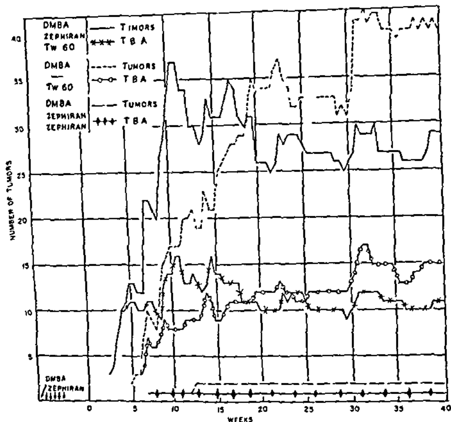


Fig 5

ness resulted this time and new crusting was only occasionally seen Furthermore many mice had hair growing between the epilated patches and stripes and on sides of the treated area The animals were then left to heal the damages caused to the skin and a growth phase developed on the back every animal Hair first continued to grow vigorously in the patchy way described above, but later the growth wave faded and the sharp contrast between the epilated and not epilated patches and stripes became less conspicuous In the end of this period the treated skin was still slightly thickened but no more tender It was not as easily movable over the back as in the normal The skin of the animals killed at this time showed only slight hyperplasia, some increase of fibres of the connective tissue and occasionally deformation of follicles (Fig 3)

The Tumor Yield and the Histology in the Period of the Final Treatments

The tumor yield in the different groups is given in Fig 5 The group pretreated with initiation and 6 4 per cent Zephiran before Tween 60



Figs. 1–4

Fig. 1. Skin of a mouse 3 days after the first treatment with 0.4 per cent watery solution of Zephiran. Degenerative alterations of the epidermal and follicular cells. Many inflammatory cells particularly in the deeper dermis. × 150.—*Fig. 2.* Skin of a mouse after 3 treatments with the same solution as above. Necrosis of the epidermis and of the infundibular parts of the common follicles. Abundance of inflammatory cells mainly polymorphonuclears in all layers of the epidermis. × 70.—*Fig. 3.* Skin 3 weeks after the last or 5th application of the 0.4 per cent watery solution of Zephiran. Slight hyperplasia of the epidermis and increase of the cells and fibres of the dermis. × 150.—*Fig. 4.* Skin after 10 weeks of promoting treatments in the init. Zephiran Tween 60 group. A particularly strong hyperplasia of epidermis and of the walls of the common follicles. Abundance of young connective tissue cells and inflammatory cells in the dermis. × 70.

he Experiment

...al of ...ors	TTl animals	Tumors studied histologically								Average latent period to the appearance of the 1st tumor
		Before the end of the experiment				At the end of the experiment				
		Tot No	Papilloma	Acanthoma	Ca	Tot No	Papilloma	Acanthoma	Ca	
29	2 6	10	10		–	18	13	5	–	8 7 weeks
2	2 0					2	2			7 0 weeks
40	2 7	1				12	11	15	6	14 4 weeks

in the group treated Initiation, Zephiran, Tween 60 Such tumors were called acanthomas in accordance with a classification worked out in this laboratory (5)

In the groups run parallel for the histology, the animals in the group Initiation, Zephiran, Tween 60 showed very strong hyperplasia of the skin especially during the time of the vigorous tumor development in this group Because of remarkable thickening and elongation of the walls of the compound follicles, the sebaceous glands and the hair forming parts (germinal parts) in the bottom of the follicles were very deeply located In many animals the number of the follicles per unit area in the sections simultaneously increased, so that the skin was stuffed with thick hair sheets (Fig 4) The dermal tissue between the follicles was rich in cells and bulged in a papillomatous pattern between the follicles The cells were young connective tissue cells and inflammatory cells many of them granulocytes Increase of fibres and vascularity was conspicuous These changes were most accentuated in the animals killed after 10 and 20 weeks of treatment In the last 2 animals of the histology group and in those which were killed in the end of the experiment (40 weeks) the dermis however showed reduction of fibroblasts stiffening and hyalinisation of the collagenous bundles (Fig 6) The dermal connective tissue was consequently converted to a scarlike tissue with paucity of cells and vessels

In the group pretreated only by initiation the hyperplasia did not reach the same extent as in the former group during the first half period of promotion Later there was no clear difference, but instead the dermal connective tissue was considerably better preserved than in the former group The thick collagen bundles were still arranged in a network and the inflammatory reactive changes were abundant

TABLE
Results at the Fi

Treatment	No of ♀ Swiss mice	Survivors at			Tumor bearing animals
		20 weeks	30 weeks	40 weeks	
DMBA 20 µg in acetone once—5 treatments with 2 4 mg Zephiran in a 6 4 per cent watery solution—Tween 60 daily	30	26	21	16	11 36 7 per cen
Ditto, but in stead of Tween 60, 0 6 mg *Zephiran in a 3 2 per cent watery solution* trice weekly	30	29	25	21	1 3 8 per cen
DMBA 20 µg in acetone once—Tween 60 daily	30	29	24	22	15 51 7 per cen

developed papillomas very quickly Over 55 per cent of the effective total number of animals had tumors after 11 weeks of treatment and the average latent period to the appearance of the first tumor in each tumor bearing animal was 8 7 weeks The total number of tumors as well as the number of tumor bearing animals started to diminish after the 12th week, but the reality of that trend is difficult to assess, because of increasing mortality rate among the animals during the rest of the experiment Instead the group pretreated only with DMBA before the start of promotion with Tween 60 developed papillomas some weeks later and the increase of the number of tumors and of tumor bearing animals did not follow so quickly but instead more persistently than in the former group After 11 weeks 27 per cent of the effective total number of animals had tumors and the average latent period was 14 4 weeks Around the 18th week the tumor rate of the former group was overtaken See Fig 5—Only one animal in the group pretreated by initiation and 6 4 per cent Zephiran and followed by 3 2 per cent Zephiran continuously thrice weekly, got tumors 2 pedunculated papillomas appeared relatively early on the treated skin They grew continually and become finally keratinized on the top, but remained all the time benign The animal was exceptional big and strong The skin of the other animals in this group became early dry and covered with yellow squamas

When the animals were killed after 40 weeks of final treatment and histological study was made of all tumors found in the different animals, only the group which was not treated with Zephiran at all had carcinomas The number of them was 6, two of which were basocellular (see Table 1) But many other tumors too in this group were more rich in epithelium and displayed more often atypicalities than the tumors

throughout the dermis when the animals were killed in the end of the experiment (Fig 7)

The group treated by Initiation, Zephiran, Zephiran showed during the period of final treatment moderate to good hyperplasia of the epidermis, but already after one month signs of degenerative changes of the collagenous bundles, scarcity of fibroblasts, inflammation and vascularity The dermis became finally sclerotic and devoid of any fresh reactive changes (Fig 8) Significantly the dermis of the single tumor bearing individual, was definitely in better condition than the corresponding structure of the other animals in the same group

The regressing papillomas encountered among other tumors in the experimental groups all showed circulatory disturbancies and stromal deterioration

DISCUSSION

Studies concerning the effect of damage and repair has been actual in the experimental approach on skin tumorigenesis since the work of *Deelman* 1923–1924 Wound healing has been found to be a good promoter of tumors in the skin of rabbits (9, 10), but in the instance of mice there has been no clear cut evidence of the effect of such a situation (8, 13, 14) Ischemia caused by arterial occlusion enhanced tumor formation in the ears of rabbits (15), but subsequent to adrenalin injections it did not promote tumors in the skin of mice (16) The known chemical promoters all are irritants The mode of action of them has, however, been estimated very differently (1, 12, 18) *Friedewald & Rous* (1944) defined the histopathological equivalents of promotion in terms of hyperplasia, inflammation hyperaemia and connective tissue participation

The comparative histological study of the changes in the skin and the corresponding tumors helped to substantiate general pathogenetical conditions in the groups studied The comparison also produced evidences indicating more essential differences between the groups, than was given by mere numbers of tumors As in earlier studies (2, 7, 11) the tumor rates were again directly proportional to the hyperplasias, but this time it was directly proved that the degree of hyperplasia was working only if the dermis was not regressively altered by the action of the promoter When that happens, even a good hyperplasia is insufficient to bring out tumors In fact, the pertinent changes of the stage of promotion of papillomas of mice are the same as noted by *Friedewald & Rous* to be existing at the stage of promotion of the papillomas of rabbits The changes must only be produced under so long run of time as possible without appearance of apparent regressive alterations The last point might be the most important fact by judgement if a irritant is a promoter or not

No carcinomas appeared in the group, which was treated with the deeply damaging Zephiran doses before the final Tween 60 treatments



Fig 6

Skin of a mouse in the same group as in Fig 4 after 40 weeks of promoting treatment Still a remarkable hyperplasia of the epidermis but considerable regressive alterations and scanty of inflammatory changes in the dermis × 100



Fig 7

Skin of a mouse in the group init — Tween 60 after 40 weeks of Tween 60 treatments daily Strong hyperplasia of the epidermis good preservation of the follicles and of the fibrous structures of the dermis Considerable amount of fibroblasts and inflammatory cells in the dermis × 100

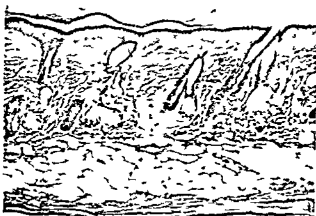


Fig 8

Skin of a mouse in the init Zephiran Zephiran group 40 weeks of Zephiran (0.2 per cent watery solution) treatments thrice weekly Rather good hyperplasia of the epidermis but dermis scarlike and poor of cells and vessels The condition of the common follicles does not seem to be healthy × 100

week The same dose of phenol, but given in a 5 per cent solution alternatively in the anterior and posterior halves of the back, caused no ulceration, but transient crusting and was found to have only moderate promoting effect confined to development of papillomas It is obvious, that the former form of treatment elicited more effectively the type of reparative and progressive process useful in promotion than the latter

Recognition of conditions, like the regressive sclerosis of the dermis, makes it conceivable, that situations would arise, where epidermis is strongly induced with neoplastic potentialities, but the dermal environment of a kind, that tumorous growth is impossible This might be the case for instance in chronic radiodermatitis, many precanceroses and carcinoma in situ

SUMMARY

1) The effect of 5 ulcerating doses of 6 4 per cent watery solution of alkyldimethylbenzylammoniumchloride (Zephiran) on the outcome of a promoting experiment, where mice were initiated with 20 micrograms of DMBA in acetone and promoted with polyoxyethylene sorbitan monostearate (Tween 60) daily, was studied The treatment with Zephiran, dispersed over 2 weeks, was began one week after the initiation, and it aimed to cause persistent regressive changes in the dermis *before* commencement of the promotion Slight fibrosis of the dermal collagen ensued when the lesions healed The promoting effect of a 3 2 per cent watery solution of Zephiran given thrice weekly was studied too The tumorigenetical conditions of the skin were examined histologically in parallel series of mice Autopsies were performed and samples taken after 1, 2, 4, 10, 20 and 40 weeks of treatment All available tumors were subjected to [illegible]

2) The 5 interm [illegible] the promotion effect of Twee [illegible] the appearance of papillomas

3) The enhancement due to this Zephiran effect was seen early during the course of promotion and it was directly related to exaggeration of epidermal and follicular hyperplasia, to increase of young connective tissue cells and inflammatory cells and to accentuation of hyperaemia as compared with the corresponding changes when the preparatory Zephiran treatments were omitted

4) These changes of hightened reactivity were later during the promotion converted in sclerosis of dermal collagen and in a general involution of progressive changes The incidence of papillomas seemed to decline and no carcinomas occurred contrary to the group lacking the Zephiran intervention

5) A weaker Zephiran solution in a comparable situation was unsuccesful as promoter inspite of hyperplasia adequate for tumor development When a regressive dermal condition here appeared very early in the course of the treatments, the conclusion gained strenght,

If that step was omitted, 6 carcinomas and a lot of acanthomas occured. This is interesting from the point of view, that there is a opinion claiming that regressive changes would be prerequisites for the development of malignancy. It is well known, that for instance elastoid or basophilic degeneration of the collagen is very often coexistent with malignant tumors in the skin of face and other exposed areas of human beings. Could this difference in the quality of the tumors in the experimental groups depend on mere inadequate experimental conditions? The groups did not differ so much in size during the last 10 weeks of promoting treatment, that it would have been impossible to develop carcinomas in the Initiation, Zephiran, Tween 60 group. An other explanation for this difference would be that the ulceration and desquamation of the skin caused by the 5 intermediate Zephiran treatments, could have reduced the initiated elements of the epidermis and follicles to such an extent, that development of malignant type of growth later was impossible. Also this argument seems to be inconclusive in view of the abundant occurence of papillomas during the first 10 weeks of subsequent promoting treatment with Tween 60. Finally Zephiran itself was not anticarcinogenic because it could sensitize the skin for a faster promotion of tumors than was possible without this intervention. It is therefore obvious that the difference in the quality as well as in the final quantity of the tumors, was dependent on the regressive alterations of the dermis caused by Zephiran.

It seems to be much more difficult to reach a suitable, presevative and long lasting repair reaction in the skin of the mice than it is in the skin of the rabbit. The reactive capacity inherent in the skin of the mouse is apt to be easily overstressed and exhausted and the productive changes are soon turned over to regressive features. Consequently benign papillomas dependent on the productive state of the dermis, are vanishing or they never get opportunity to manifest themselves.

There is evidences in the literature, that establishment of progressive dermal changes do have direct bearing on the situation of the genesis of papillomas. In the experiments of *Linell* 1947 (9) it was first after the dermis of the ear of rabbits, changed by long term tar paintings, had been transformed by the way of deep punching to a granulation tissue, that papillomas appeared at the healed punch hole. A superficial trauma made out by rubbing off the epidermis, was ineffective although it ensued in at least as much hyperplasia as the procedure of punching. The situation in the experiments of Salaman and Glendenning, who used mice, certainly is closely related to the punch hole technique. They reported development of papillomas and some carcinomas after initiation with 0.2 ml of 0.15 per cent DMBA in acetone and promotion with 20 per cent phenol in acetone. One application of this phenol caused transitory toxic symptoms and local ulceration of the skin which healed about 3 weeks later. Consequently, in the tumor induction experiment the phenol was given on four places in rotation, one application each

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A HISTOLOGICAL AND CYTOLOGICAL STUDY OF DIFFERENT METHODS OF SKIN TUMORIGENESIS IN MICE

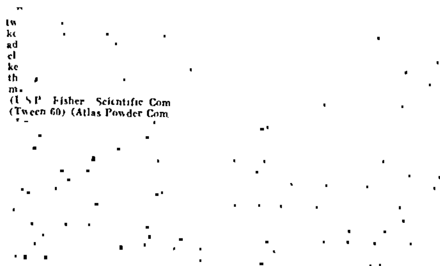
By

KAI DAMMERT[1]

Received 14 i 61

The pathogenesis of experimental skin tumors has been studied histologically by many workers (3, 4, 6, 9, 10, 11, 13, 15, 16, 19 and 28) contemporarily with the development of the methods of tumor induction. Attention has been paid to all structures of the skin but still the exact relationship between preneoplastic changes and the later developing skin tumors of different morphological and biological types is obscure. In the present study detailed histological and cytological studies have been carried out to compare and contrast the early changes seen with different methods of carcinogenesis. In particular studies of mitotic frequency have been compared.

MATERIAL AND METHODS


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(Tween 60) (Atlas Powder Com

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that the regressive dermal changes were incompatible with the development of papillomas as well as of carcinomas

6) Epidermal hyperplasia was consequently found to be important in papillogenesis only if supported by pure and long lasting progressive and inflammatory changes of dermis

7) The regressing papillomas showed stromal deterioration

8) The results are discussed

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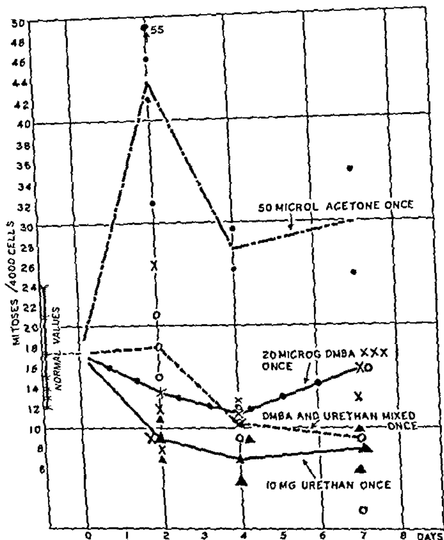


Fig 1

Mitotic c unts after one applicati n carcinogens and acetone used as solvent The applicati n f s lvent alone di l rise the mitotic *frequency* whereas the carcinogenic chemicals tended to depress it

were marked especially two days after the applications The treatment with acetone was followed *by considerable* increase of the mitotic ac tivity The mitotic counts in the *groups* treated with DMBA, urethan and the mixture were, with the exception of one animal in the DMBA group, for the most part *inside the lower* limit of the normal range or below it In comparison with the *solvent group* these last groups showed an obvious inhibition of the *mitotic activity* After seven days this effect

hematoxylin and eosin and prepared from each animal was subjected to counting. Sometimes it was necessary to control the counting with a second section. In each section the number of mitoses among 1000 epidermal cells was counted for a total of 4000 cells for each animal. The examination was done with oil immersion at a magnification of 1000 ×.

The fixation in Susa is particularly suitable for observation of cell division in all stages. Only cells with clear mitotic features were considered in mitosis. The earliest changes considered pertinent to the beginning of the division were loss or fading of the nuclear membrane and nucleoli with appearance of chromosomes. The method of ordinary sectioning was not very suitable for the study of mitotic abnormalities. However, abnormalities such as clumping and stickiness or spread of the chromosomes was fairly often seen and therefore a reliable distinction between pro and metaphases was difficult. Since there seemed to be no clear deviation from the normal sequence and timing of the different mitotic stages in any of the experimental groups, in final results no attention was paid to the relative frequency of the separate stages. After separation of the daughter cells and restitution of the nuclear membrane, the mitotic division was considered finished. When hesitation arose whether a cell with a pyknotic nucleus was actually in mitosis or not, it was not included in the count and considered a degenerating cell.

In order to limit the test to qualitatively similar tissues, only interfollicular epidermis was included in the examination. According to *Pinkus* (20 pp. 20–21), the pilosebaceous apparatus ends at the surface of the skin with an intraepidermal "infundibular unit" which differs from the epidermis proper.

In the figures to be presented with the experiments, the arithmetical mean values for the different animals examined at the same time are given. In Fig. 1 the individual results are also reported. For the evaluation of the epidermal hyperplasia caused by the different treatments the number of cell layers was estimated by viewing the four sections for the whole treated area and giving the main impression in two figures for each section. From the eight values obtained an arithmetical mean value was computed.

EXPERIMENTS AND RESULTS

Untreated Animals

The skin of nine animals, shaved one week before killing, was examined. The epidermis consisted of one or two layers of rounded or cylindrical cells, surmounted by few flat granulated cells here and there and by a tiny lamellary stratum corneum. In the basal layer regular mitotic figures were found, corresponding to a mean rate of 17.6 with a standard deviation of 4.5/4000 cells. The dermo-epidermal junction and the close vicinity of follicles was made up by fine argyrophilic fibres, which with the PAS stain appeared more homogeneous, suggesting the presence of PAS-positive ground substance among the fibres. Some histiocytes and mast cells were present mostly around the capillaries and the follicles.

Experiments with Single Treatment

Four groups of animals were treated only once as shown in Fig. 1. The application was made with a calibrated micropipette spreading the solution evenly to the shaved area. In each group 2 to 4 animals were sacrificed at the 2nd, 4th and 7th day after treatment. Fig. 1 shows the results of the counting of the mitotic cells in the different individuals and the curves drawn according to the values. The individual variations

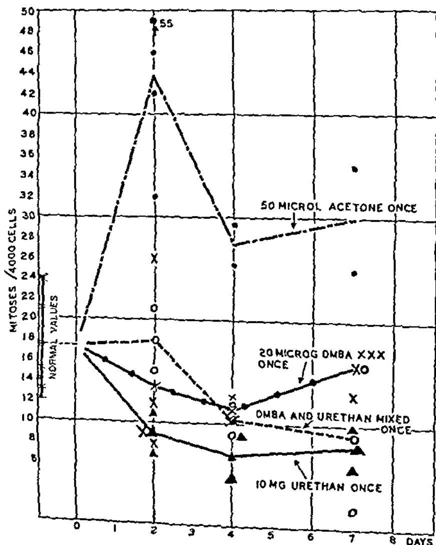


Fig. 1

Mitotic counts after *one application* carcinogens and acetone used as solvent. The application of solvent alone did rise the *mitotic frequency* whereas the carcinogenic chemicals tended to depress it

were marked especially two days after the applications. The treatment with acetone was followed by considerable increase of the mitotic activity. The mitotic *counts* in the groups treated with DMBA, urethan and the mixture were, with the exception of one animal in the DMBA group, for the most *part inside* the lower limit of the normal range or below it. In comparison with the *solvent* group these last groups showed an obvious inhibition of the *mitotic* activity. After seven days this effect

was less appreciable in the DMBA group than in the urethan and the mixture group

The different treatments did not cause any clear hyperplasia, but a slight increase of the number of cells were seen after seven days in the group treated with acetone alone. In the DMBA group and the group treated with the DMBA-urethan mixture many of the mitotic cells showed signs of acute nuclear damage of the pyknotic type 2 and 4 days after the treatment. At the same time irregularities in the shape and size were obvious, but substantial enlargement with even giant epithelial cells were seen only in the DMBA group. Minor mitotic abnormalities were observed in the group treated with urethan. In the mixture and urethan groups the epidermal cells were generally smaller and more basophilic than in the DMBA and acetone groups. All treatments caused slight inflammatory changes and some swelling of the collagenous bundles in the dermis. These changes were more marked in the groups treated with DMBA and the mixture. No special differences from the normal were seen in PAS and Snook preparations.

TABLE 1

Treatment	No of ♀ Swiss mice		Time of sacrifice in weeks*				
			1/2	1	2	4	10	20
			Number of mitoses per 4000 cells					
DMBA 20 μg in 50 μl acetone once	19		21	25	25	34	46	22
			19	21	18	15	24	41
			18	23	27	22	20	27
			16					
		Average	18.5	23	23.3	23.7	30	30
DMBA 20 μg in 50 μl acetone once followed a week later by Tween 60	17		55	47	60	34	50	24
			91	42	44	43	28	22
				37	58	45	33	
		Average	73	42	54	40.7	37	23
Acetone 50 μl once and shaving when necessary	7					21	26	26
						29	32	24
						18		
		Average				22.6	29	25

Initiation Promotion Experiments

Experiment A.—A group of 19 animals were treated with 20 μg in acetone once, a second group of 17 received the same primary treatment followed a week later by Tween 60 daily, a third group of 7 animals received only acetone once. DMBA was applied with a micropipette as in previous experiment. Tween 60 was applied with a standard glass dropper each drop measuring approximately 20 μl, beginning one week

* Starting one week after treatment with DMBA or acetone

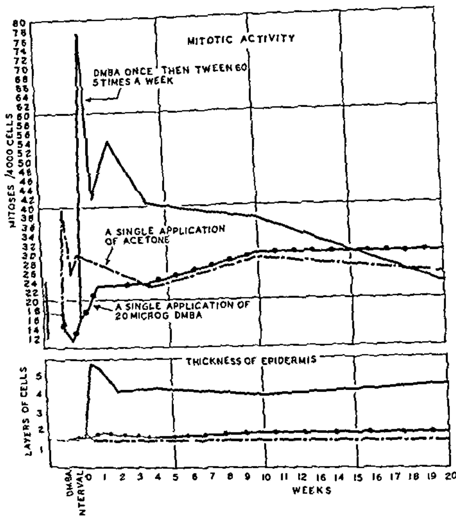


Fig 2

The mit tic frequency in relati n t ... le el ... men ... f h ... stage experiment with one ap ... lvent and the pr moter Twee ... for limited periods The pr ... ia of the epidermis in spite ... e of the experiment

after the initiation Two *drops* were spread to cover the whole experimental area Table 1 shows the distribution of samples taken in connection with the autopsies and the results obtained by counting of mitoses in different individuals The results in terms of the mitotic rates and number of layers of cells in epidermis are illustrated in Fig 2 In this chart the initial depression observed in the previous experiment after the single dose of DMBA was again represented It was followed by



Fig 3
Left a patch of epidermis 20 weeks after one application of 20 µg of DMBA in acetone Enlargement of the nuclei and slight variation of size and polarity of the cells Right epidermis of normal appearance 20 weeks after one application of the solvent H & L × 730

a rise which remained steadily above the normal level for the remainder of the period of study, that is up to 20 weeks However, the rate observed after the application of the solvent alone with periodical clipping of the hair did not differ in any significant way

While the cells of the epidermis mainly increased in size during the first week after the single dose of DMBA, some trends to slight hyperplasia persisted for the rest of the experimental period The scanty thickening of the epidermis seen was not of any generalized type, but patchy and partially due to the hyperplasia and a slight increase in size of the cells and hyperkeratosis Some of these patches showed at the end of the experiment cells with conspicuous variation in shape and size and a particular increase of the size of nuclei (Fig 3) In such places a subepidermal loosening of the dermal collagenous tissue often was an additional finding This was combined with coarsening and fragmentation of the reticular fibers and with an increase of PAS reactive substance especially in the dermo epidermal junction The group given acetone once and kept free of hair with periodical clipping showed skin structures of normal appearance

The addition of continued promoting treatment resulted in a huge, immediate rise in the mitotic frequency accompanied by inflammatory changes with hyperplasia and hypertrophy of the epidermal cells and an increase in dermal tissues However, the peak of the rise in the mitotic frequency dropped rapidly and continued to decline in spite of

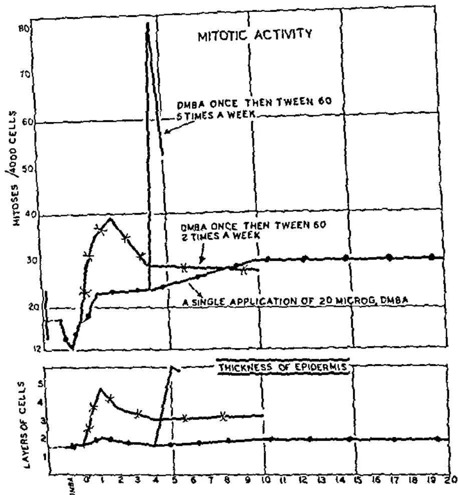


Fig 4
The mit tic frequency in relati n t hyperplasia after one application of DMBA foll wed by Tween 60 twice a week and five times a week When Tween 60 ... given five times a ... hyperplasia and mi ... ficantly higher tha ...

the continuous treatment Despite ... of hyperplasia was maintained a ... began to develop under these con ... weeks had small papillomas in th ... One of the last 3 killed after 20 weeks of continual promoting treatment had a rather big pedunculated papilloma in the area under examination

Already after 4 weeks of treatment the epidermo dermal junction was broad and rich in reticulum fibres *and* PAS reactive materials The affinity to PAS stain extended later to involve the whole dermis

However no substantial derangement in the dermal collagenous structures was seen

Experiment B —5 animals were treated with 20 μg DMBA in acetone once and after 4 weeks with Tween 60 daily 15 animals received the same preparatory treatment with 20 μg DMBA followed a week later by Tween 60 twice weekly Table 2 shows the distribution of samples and the results of the mitotic counts in the individual animals Fig 4 shows the pertinent curves of mitotic activity and the related hyperplasia Accordingly, when the Tween 60 treatment was started four weeks after the single application of DMBA, the epidermis reacted to the promoting treatment with Tween 60 with the same degree of mitotic increase and hyperplasia as after one week of interval When Tween 60 was applied only twice a week, the increase of mitotic rate and hyperplasia were significantly less than with daily treatments Also in the experimental group that received Tween 60 twice weekly for 10 weeks, one of the last three animals had two small papillomas Both groups had abundant inflammatory changes and thickening of the dermis, more pronounced in the group with five than with two weekly treatments

TABLE 2

Treatment	No of ♀ Swiss mice	Time of sacrifice in weeks*					
		1 2	1	2	4	10	20
		Number of mitoses per 4000 cells					
DMBA 20 μg in 50 μl acetone once followed 4 weeks later by Tween 60 five times weekly	5	93 56 53	53 52				
	Average	77 4	52 5				
DMBA 20 μg in 50 ul acetone once followed a week later by Tween 60 twice weekly	15	30 31 26	39 33 33	38 42 38	39 22 26	22 34 27	
	Average	29	35	39 3	29	27 7	

Experiments with Repeated Applications of DMBA or Urethan or a Mixture

In this experiment four groups of animals were treated as follows a group of 19 animals received 1 μg DMBA in acetone, a second of 18 animals 4 mg urethan in acetone and a third of 19 animals DMBA and urethan together in acetone A group of 17 animals received acetone only The acetone solutions were applied with a regular glass dropper The concentration of the DMBA solution was 0 005 per cent and that of the urethan solution was 20 per cent The mixed solution contained 0 005 per cent DMBA and 20 per cent urethan Each animal received

* Starting from the beginning of the treatment with Tween 60

twice weekly one drop of approximately 20 μl of the respective solutions

The quantitative aspects of mitotic activity and hyperplasia are illustrated in Table 3 and Fig 5 DMBA and especially urethan depressed the mitotic activity as compared with the effect of the solvent alone Urethan added to the DMBA caused a mitotic rate which was between the rates of DMBA and urethan alone As can be seen from Fig 5, the mitotic activity was not paralleled by corresponding hyperplasia The significant increase of the mitotic rate caused by the repeated application of solvent was not followed by any apparent hyperplasia The relative increase of mitotic activity seen with repeated DMBA treatments resulted in hyperplasia only after the 10th week, whereas the low rate of mitoses observed in the mixture group, was matched to a hyperplasia by a continuous increase of cells which began already at the 4th week A few of the mice treated with urethan alone had patchy areas of hyperplasia, these were only seen in the first month of study and were not consistent Two animals of the group treated with the mixture developed broad based warts during the later part of the experimental period

The histological features pertinent to different groups were as follows

TABLE 3

Treatment	No of ♀ Swiss mice	Time of sacrifice in weeks* 1 2	1	2	4	10	20
		Number of mitoses per 4000 cells					
DMBA 1 μg in acetone twice weekly	19	16 18 28 32	14 24 20	17 20 20	35 24 20	30 27 20	35 32 27
	Average	23 5	19.3	19 9	26 3	25 7	31 3
Urethan 4 mg in acetone twice weekly	18	14 19 17 17	20 19 12	7 21	16 21 14	18 16 14	11 19 19
	Average	16 7	17	14	17	16	16 3
DMBA 1 μg mixed with Urethan 4 mg in acetone twice weekly	19	16 24 20 20	10 22 22	27 10 16	15 23 12	17 17 21	20 17 20
	Average	19	18	17 7	16 7	18 3	19
Acetone twice weekly	17	32 24	27 29 22	25 30 33	44 36 35	33 30 31	35 36 29
	Average	28	26	23	38	31 3	33 3

* Treatment given continuously for 20 weeks

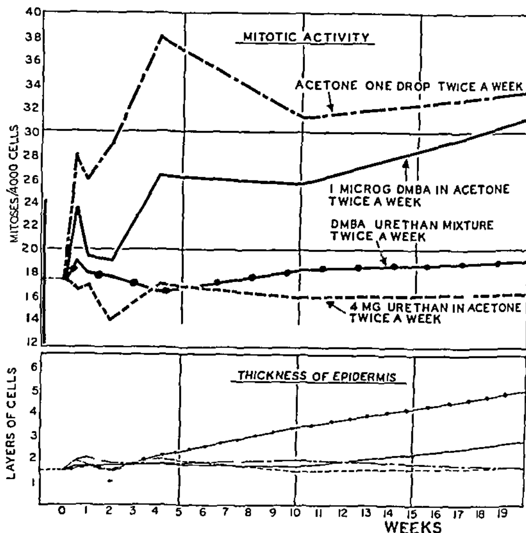


Fig 5
Acetone paintings twice a week was causing slight increase of the mitotic activity DMBA and particularly urethan dissolved in it did have an inhibitory effect on the mitotic frequency In spite of that the mixture of DMBA and urethan elicited a *continually increasing epidermal hyperplasia*

Repeated application of 1 µg DMBA uneven hyperplasia, variations in shape and size of the cells, irregularities in stratification of the epidermis, increasing basophilia and nucleocytoplastic ratio, hyperkeratosis In the dermis there was some infiltration of lymphocytes, histiocytes, mast cells and fibroblasts and a thinning of the collagenous bundles Subepidermal patches with increased affinity for PAS and silver stain was shown after the 10th week of treatment A considerable degree of individual variation was apparent

Repeated application of 4 mg urethan minimal patchy hyperplasia in some animals during the first month of treatment Enhanced resistance of epidermal cells against intracellular fluid and a trend toward accumulation of keratin on the surface In some spots a slight inflam-



Fig 6
Left ep dermis of a mouse after 20 weeks of repeated treatments with 4 mg urethan Enlargement of the size and especially of the nuclei of the cells Right normal looking epidermis after 20 weeks of repeated treatments with the solvent
H & E × 730

matory reaction From the 10th week onwards cytological abnormalities began to develop these included an increase in cell size and nucleocytoplastic ratio enlargement of the nucleoli and coarsening of the chromatin pattern of the nuclei as compared with the skin of the mice treated with solvent alone (Fig 6) Alterations similar to those caused by DMBA in connective tissue were seen in the skin of the mice treated during 20 weeks

Repeated application of the DMBA urethan mixture Accentuation of changes seen with DMBA alone such as increasing basophilia of the cells ensuing in a continual accumulation of cells of basal or stem cell type On the other hand an enhanced keratinisation of the same cells in the upper layers of the epidermis Continually increasing cellular and histological abnormalities and loss of coherence between the cells and between the cells and connective tissue in the dermo epidermal junction (Fig 7) Here an increase and fragmentation of the reticular fibers was observed from the 4th week onwards This feature seemed to develop paralel to increasing stainability with PAS The dermo epidermal junction was eventually converted to an indefinite borderline zone With the advance of time the dermis became more and more thick and rich in cells The original wavy meshwork of collagen bundles was continually changed to a fine fibred less organized type of connective tissue The alteration was again accompanied by increasing affinity for silver stain and PAS stain



Fig 7
Epidermis of a mouse after 20 weeks of repeated treatments with DMBA-urethan mixture in acetone Note the conspicuous variation of the size and polarity of the cells and the loss of the cohesiveness between them H & E × 730

DISCUSSION

Chemical carcinogenesis in the skin of the mouse may be modified in different ways Thus tumors can be induced with chemical carcinogens applied repeatedly or once in a very large dose again tumors may be induced by using a single initiating dose of a carcinogen followed by a promoting agent such as Tween 60 or croton oil Under these different conditions not only do the results differ in terms of numbers of tumors induced and time taken for induction, but additionally the types of tumors induced are different (26) In other studies it has been found that the action of one carcinogen can often be partially inhibited by the simultaneous application of another carcinogen (22) In recent studies in this laboratory it has been reported that addition of urethan to DMBA for skin applications will delay the effects of the latter carcinogen (27) On the other hand some synergistic action of these two chemical was demonstrated when they were used together as initiators (12) The understanding of these seemingly opposite results seemed to depend on a better knowledgement about the basic tissue changes relevant to initiation or repeated use of chemical carcinogens respective promotion

As the results are now, there was considerable individual variety of the mitotic frequencies in each group of experimental animals The use of an inbred strain of mice would sure had eliminated the most disturbing extremes Increases of the amount of animals had perhaps made a statistical evaluation of the results possible By now there was however satisfactory significance of difference between the solvent groups and

the groups treated with DMBA and/or urethan and between the latters and the groups exemplifying the 2 stage theory of tumorigenesis

The mitotic activity of the epidermis was different in the different experimental groups DMBA and urethan had primarily a depressive effect on the mitotic frequency, less effective in cases of DMBA than in cases of urethan, when they were given repeatedly In case of one initiating dose of DMBA the depressive effect lasted only one week

The results concerning the effect of urethan were consistent with earlier findings It has been found to decrease the mitotic activity in the germinative zones of the Lieberkuhn glands of the small intestine during 24 hours after injection of urethan intraperitoneally to mice (*Dustin* 1947) (8) and to decrease the mitosis in the Walker rat carcinoma (*Boyland & Koller* 1954) (2) Different types of mitotic disturbances

As to the effect of DMBA, it has to be stressed that at no time the mitotic frequency was evidently higher with DMBA than with the solvent alone Deletary immediate effect of the carcinogenic hydrocarbons on mitosis has been reported (*Ahlstrom Ludford, Druckrey & al*) (1, 18, 7) and they have been shown to inhibit the growth of normal and tumor tissue (*Haddow & al*, *Lees*) (14, 17) Haddow et al communicated inhibition of mitosis as well (p 470) The toxic, destructive effect exerted by them has been claimed to be directly related to their carcinogenic action (*Wolbach, Graffi*) (28, 13) On the contrary, many workers have noticed a marked stimulatory effect of the same compounds *Reller & Cooper* (21) using total mount preparations of the ears of mice obtained results which showed a stimulatory effect on cell division in the epidermis within the first 48 hours after a single application of a 0 6 per cent solution of MC in benzene *Iversen & Edelstein* (16) made similar experiments with similar results using DMBA and DBA as 0 5 per cent solution in chloroform and benzene In another experiment *Cooper* and *Reller* (5) applied the same dose of MC as in the experiment with a single application but repeatedly twice a week and observed again an increase of the mitotic frequency during the experimental period of 14 weeks The increase, however, had a biphasic pattern with a temporary remission between the 37 and 65 days Recently results suggesting a comparable stimulatory effect of DMBA are reported by *Daoust* (6) after application of the carcinogen as a 0 5 and 0 25 per cent solution in benzene and by *Gimmy* (10, 11) in rabbits using 0 …

… effect This was followed by a long lasting period of repair which began with an accomodation of the epithelium to renewed applications of the carcinogen and ensued in and

excessive regeneration with vigorous mitotic activity in the follicular structures of the peripheral area

It might be difficult to explain the fundamental differences regarding the mitotic rate between the results obtained by different workers. Different strains and sometimes different species of animals have been employed. Irritative solvents such as benzene and chloroform have been used with very little control of the effect of the solvent alone. Finally, histological technique, sampling and the criteria of including cells in the count might differ very much. These workers do not give many methodical descriptions especially on the last point. Interesting is the question of the dosage level, which has direct bearing on the toxicity of carcinogenic hydrocarbons. That used for DMBA in this study is very low, yet effective enough for successful production of tumors. Such gross or microscopic destructive lesions were not observed as often is seen after larger doses of carcinogenic hydrocarbons. It is possible that the specific histopathological changes caused by the carcinogens are more easily detectable under the conditions of low dosage and using as little irritating solvents as possible, and that the rise of mitotic frequency after higher doses is of non-specific character or related to the promoting activity of the carcinogens only. It is surprising that pure acetone, that is considered to be one of the most indifferent solvents, could raise the mitotic rate of the epidermis so significantly.

It seems to be a point of great interest that the development of hyperplasia is not dependent on the mitotic activity. This is shown by the fact that the increased mitotic activity with repeated applications of acetone did not result in hyperplasia, whereas the very low mitotic activity with DMBA-urethan mixture showed finally a very high degree hyperplasia.

The special mechanism of the development of hyperplasia involved in the last case was first shown by *Glucksmann* (9) to take place during benzpyrene carcinogenesis in mice. Working with weekly applications of 1 per cent benzpyrene solution in acetone he noticed that although there was some increase of the mitotic activity the hyperplasia arose as a result of the delay in the onset of differentiation of the cells. The cells of epidermis consequently increased through a delay in their maturation process. Later in the study of the mechanism of tumorigenesis using initiation and promotion technique the induction of the delayed maturation was shown to be specially related to the action of the initiator (4 19). It seems that at least one important part of the specific action of the carcinogens is from the very beginning of the action to create cells, which are more resistent and independent than normal cells. The ability to delay the maturation might be one indication of this type of new biological property of the cells. This special type of formative change seems to be running parallel with other preparatory processes in the dermis.

In the case of active promoting treatment the establishment of a high degree of hyperplasia followed a different pathway. It coincided with

strong proliferative activity, although the later maintenance of the hyperplasia was no longer dependant on it The state of hyperplasia then persisted with a cellular exchange balance of almost normal rate This implies that a sort of accomodation toward the influence of Tween 60 must have taken place on the part of the cells The same was the case although without the same degree of hyperplasia, when Tween 60 was applied only twice weekly The differences in the degree of hyperplasia certainly are connected with the better tumor production effect of daily applications of Tween 60 than of twice weekly applications of the same The results is in keeping with earlier findings considering the significance of hyperplasia during promotion (4, 19) However, the fact that the papillomas in these initiation promotion groups did not appear correlated to any new peak of mitotic activity or hyperplasia, contradicts the decisive role of hyperplasia for the development of papillomas during promotion

There is a good evidence that the initiation promotion procedure usually is inducing papillomas and the repeated use of carcinogens again a considerable number of carcinomas (26, 4) The corresponding differences in the preneoplastic tissue reactions elicited by the respective methods are suggesting somewhat different pathogenesis in case of papillomas than in the case of carcinomas

Urethan applied in acetone in much higher doses than in this experiment did not cause any histological changes in the experiment of *Salaman & Roe* 1953 Neither did *Ritchie* (23) see any epidermal changes *after* a single or *10* injections of urethan although subsequent topical applications of croton oil were able to elicit many times as many papillomata per survivor as *could croton oil* applied to unprepared mouse skin Some animals in the experiment made now had occasionally during the first month areas of hyperplasia on the treated skin but otherwise the thickness *of the epidermis* remained normal In the cells, however both nuclear and cytoplasmic changes with features similar to those caused by repeated use of DMBA were seen With them the relative increase of nuclei and nucleoli, further hyperchromasia and cytoplasmic basophilia is ment It can be mentioned that some animals in a parallel tumor production experiment had conspicuous epidermal hyperplasia as late as 35 and 40 weeks of treatment

In the light of the most pronounced delay of maturation of the epidermal cells and the many preneoplastic abnormalities of the tissue added to the picture of DMBA action by urethan the innermost character of the inhibitory effect of the urethan on the tumor producing capacity of DMBA is obscure The morphological findings now obtained indicate that the inhibition might not be dependant on any loss of neoplastic potentialities caused by urethan but merely by a delay of the appearance of the tumors

excessive regeneration with vigorous mitotic activity in the follicular structures of the peripheral area

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SUMMARY

(1) Histological and cytological studies were undertaken on mouse epidermis at various intervals following different chemical procedures Mice were treated with (a) 20 μg of 9,10-dimethyl-1,2-benzanthracene (DMBA) or 10 mg urethan, alone or together in acetone only ones, (b) 1 μg of DMBA or 4 mg urethan alone or as a mixture twice each week (c) a single dose of 20 μg of DMBA followed by Tween 60 either daily *or twice weekly Examinations were undertaken 2, 4 or 7 days following* the single treatments and at ½, 1, 2, 4, 10 and 20 weeks during the prolonged treatments

(2) DMBA and urethan in the doses used were found to be inhibitors of mitotic division of the epidermal cells compared with the effect of solvent alone Some minor histological and cytological changes remain in the tissue even 20 weeks after one treatment with DMBA

(3) The stage of promotion is commenced with a strong mitotic activity

(4) Hyperplasia arises in quite different ways when an initiation-promotion sequence is applied than with a repeated use of low doses of DMBA or specially when DMBA-urethan mixture is used In the first case strong hyperplasia follows as a result of a vigorous mitotic activity rapidly and without dependence of the interval after initiation, in the second case an even higher degree of hyperplasia develops but slowly and under condition of mitotic inhibition estimated from the effect of the solvent alone

5 Hyperplasia is maintained during the promotion in spite of declining mitotic rate and the degree of it seems to have direct proportionality to the effect of promotion Papillomas appear without additional increase of mitotic activity of hyperplasia which implies that skin structure other than epidermis must have a decisive role at the time of the formation of papillomas

6 The changes seen with urethan are slight but both in epidermis and dermis of the same quality as observed with DMBA Added to DMBA, it acts synergistic causing much more preneoplastic irregularities and interference of masturation of the cells than both would do separately

(7) The results are discussed

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autopsies and is well above the frequency given by most other authors especially

from stud es of literature before 1935 and after 1947 respectively and is in accordance with more recent statistics stating an incidence of 0.3 0 4 per cent (7 11 17 18 19)

Thus a general increase in the incidence of primary liver cancer is established from earlier surveys and from this material quite a considerable one

A possible cause for this higher percentage may be an increased accumulation of carcinomas in autopsies and in order to obtain a more relevant criterion for the incidence of primary carcinoma of the liver the frequency has been evaluated in proportion to the total number of carcinomas This total was 3653 for the whole period corresponding to 24 62 per cent of the 14 881 autopsies with a rise from 20 03 per cent to 27 45 per cent for the two eleven year periods a moderate increase in proportion to the above mentioned which is almost threefold

Accordingly the incidence of primary liver carcinoma in relation to all carcinomas was observed to rise from 1 83 per cent to 3.29 per cent for the two eleven year periods As compared with the corresponding figures from earlier and more recent studies a similar rise is noticed from 1 2 per cent (4 17) to an average of about 2 5 per cent respectively (8 14 17 19 20)

OBSERVATIONS

Age and Sex

Fig 1 illustrates the age incidence and sex distribution

A considerable concentration of primary carcinoma of the liver was observed within the older age groups with 81 out of 104 patients over the age of 60 The total number ranged from 35 to 86 years of age, with the majority falling in the age group between 71 and 80 years



Fig 1

According to *Berman* (4) *no age is exempt* from primary liver carcinoma which has been found even in very young children The majority of cases, however, occurred in the fifth and sixth decades *in earlier*

[1] In this connection it must be stressed that if geographical information is not specifically stated the references apply only to European and American statistics

PRIMARY CARCINOMA OF THE LIVER

A post mortem Study of 104 Cases

By

JORGEN GLENERT

Received 18.i.1961

Primary carcinoma of the liver in many ways is different when compared to other malignant growths and has, therefore, been the subject of intensive studies, in order to clarify the etiology of this type of carcinoma.

The most remarkable thing about this disease is the extreme frequency with which it appears in Africa and Asia, where it constitutes 30–50 per cent of all carcinomas (3, 4, 5, 6, 12).

In a report from the gold mines of Witwatersrand in South Africa *Berman* (4) has pointed out that this carcinoma represents 86.8 per cent of alle carcinomas among the young male Bantu negroes.

This exorbitant frequency in relation to the disease's rarity in Europe and America has resulted in numerous geographical and demographical analyses, which however all conclude that there is no special racial disposition, but that unknown factors in the environment play the decisive role.

The strange relationship between primary liver carcinoma and cirrhosis of the liver, which is found in all races, irrespective of environment, makes it highly probable that cirrhosis is a precancerous stage, and that consequently all conditions which predispose cirrhosis, must be included as possible etiological factors in primary liver cancer. From this point of view the increasing frequency of hepatitis during and after the Second World War may be important in determining the incidence of primary carcinoma of the liver.

The object of this work is to investigate this possibility, and also to analyse the data obtained from a relatively large number of autopsies with reference to the special problems concerned with primary cancer of the liver.

MATERIAL

In the period from 1938 to 1959 120 cases of primary liver carcinoma were registered in 14 881 autopsies. 104 cases were selected as being definitely primary and histologically verified.

This represents an incidence of 0.7 per cent in relation to the total number of

A similar ability of metamorphosis is demonstrated in certain types of cirrhosis where intense compensatory hyperplasia can cause the proliferating cells from one system of the liver to transform into the cells of the other system (3, 4, 17)

If such a metamorphosis can occur in cirrhosis, it is reasonable to suppose a similar reaction in carcinoma, but on this assumption it is not possible to base a classification on histogenesis

Köhn considers all liver carcinomas to originate from a common, pluripotential, undifferentiated liver cell, and the morphology of the tumour is dependent on the influence of the environment

Most authors (12, 15, 24, 25), as well as current textbooks, accept the classical differentiation between hepatocellular and cholangiocellular carcinomas, and the uncharacteristic histological structures are interpreted as the consequence of the wide range of reactivity in tumour tissue

The histological descriptions from this department often indicated the difficulty in adjusting such a narrow classification Expressions like "carcinoma, predominantly of one type, mixed carcinoma, or anaplastic carcinoma" were encountered, and occasionally "a peculiar histological form" was described For that reason a more extensive classification into the following 4 groups was preferred

(1) Carcinoma hepatocellulare, which in addition to the "pure" form also includes the carcinomas, where sporadic elements of the bile duct type are described 48 per cent

(2) Carcinoma cholangiocellulare, which comprises the typical picture as well as cases with sporadic elements of the liver cell type 34 per cent

(3) Mixed carcinomas, where the two types appear in approximately the same proportions 12 per cent

(4) Uncharacteristic tumours of anaplastic type 6 per cent

In accordance with literature the liver cell carcinoma was predominant, but the percentage was less than the 70–90 per cent found by other authors (2, 14, 25) However, all of these have differentiated between two types only

A relatively greater frequency of the bile duct carcinoma observed among women agrees with the figures of these investigators

Primary Carcinoma of the Liver and Cirrhosis

The high incidence of cirrhosis in the geographical areas where primary liver carcinoma is notoriously frequent, and the fact that 3 5–7 per cent of cirrhotic livers (25 per cent for Javanese autopsies and up to 43 per cent for African autopsies) later develop carcinoma is strong evidence of a relation between the two diseases (4, 10, 17, 19, 24)

According to *Ewing*, cirrhosis is present in 85 per cent of the hepatocellular carcinomas, and in 50 per cent of the cholangiocellular car-

investigations, and in the sixth and seventh decades in extensive recent investigations (17, 18, 19)

It further appears from Fig 1 that the disease is predominantly seen in men, the proportion of the 104 cases being 66 men and 38 women, and agrees with the figures stated by others authors (12, 17, 19)

Histology and Histogenesis

Based upon histogenetic-morphological principles, primary carcinoma of the liver is generally classified into hepatocellular and cholangiocellular carcinomas, assuming that the first originates from the cells of the liver lobules, the other from the intrahepatic bile-ducts The following table shows the most important characteristics of the two types of carcinomas

	Hepatocell carc	Cholangiocell carc
Cellular arrangement	Solid trabeculae	Alveolar tubular
Type of cell	Large polygonal with acidophilic granular cytoplasm and large irregular hyperchromatic nuclei	Uniform cubical or short cylindrical with a clear basophilic cytoplasm Hyperchromatic nuclei in one or more layers
Stroma	Sparse with numerous delicate capillaries	Abundant collagenous
Secretion	Intra and extracellular production of bile	No bile production

Although many liver tumours fit one of these descriptions, such a simple classification is inefficient, considering the variety of patterns which may occur even in one field of a single section (4) Both types of morphology may be seen in their "pure" form, but more often the two types are intermingled with a predominance of either, depending on the section where the examination is performed

Consequently many authors (4, 10, 17, 18, 19) critizise a rigid form of classification, and terms such as "mixed, combined intermediar, hepatobiliar carcinomas, and cholangiohepatomas are often used for these polymorphic tumours

Finally the histological picture may assume such a pleomorphic pattern that it is impossible to trace the true derivation

Embryologically the liver cells as well as the whole bile duct system, including the gall bladder, originate from primitive duodenal entoderm The "parent" epithelium accordingly has a potential possibility of developing into duodenal "epithelium", liver cells and bile duct "epithelium"

for the second eleven year period, the corresponding figures being 3 5 per cent from earlier literature and 7 per cent in more recent studies (2, 4, 17)

As, however, more than half of the cases of primary liver carcinoma occurred without cirrhosis the figures being 54 per cent and 57 per cent for the two periods, it can be stated that the observed rise in the incidence of primary carcinoma of the liver was caused by malignant degeneration in cirrhotic livers, but even more so in livers without cirrhosis

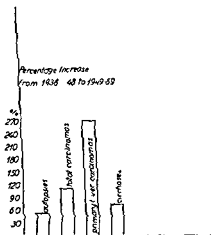


Fig 2

Fig 2 illustrates the proportionate increase from 1938–48 to 1949–59 in the number of autopsies carcinomas in all organs, primary carcinoma of the liver, and cirrhosis expressed in percentage

The figure confirms that the increased frequency of primary liver carcinoma is real and not due to the increasing number of autopsies or carcinomas of other organs The figure further illustrates that the incidence of cirrhosis has increased equally with the number of autopsies and therefore does not constitute any essential factor in the *rising* number of primary carcinoma of the liver

Gross Pathology

Generally primary liver carcinomas are macroscopically divided into nodular carcinomas and solid carcinomas, the former being characterized by a large, hard scarred and deformed liver, studded with irregular, partly confluent nodules of a whitish to greyish yellow colour The solid carcinoma often produces an enormous hepatomegalia, which is usually confined to the right lobe of the liver On section there is a polymorphic picture, with multiple smaller growths in the other lobe The colour varies according to haemorrhage, necrosis, or imbibition of

cinomas From extensive surveys of literature, *Berman* (4) and *Kohn* have found the average frequency of cirrhosis in connection with primary carcinoma of the liver to be 67 per cent The figures from the two authors' personal studies being 100 per cent and 55 per cent respectively

In certain forms of cirrhosis an enormous liver cell hyperplasia is observed with distended, almost tumour-like bile-duct formations The changing of these types of cirrhosis to a malignant form is generally accepted

In Denmark, *Krarup & Roholm* have shown that infectious hepatitis can develop into cirrhosis In view of the frequent malignant degeneration of the latter, the great number of cases of epidemical hepatitis occurring during and after the Second World War represents a special significance (17, 19, 27)

A number of authors have established a connection between hepatitis and primary liver carcinoma (1, 3, 14, 15, 22, 26) The high incidence of primary carcinoma of the liver in South Africa is considered to be caused by frequent epidemics of hepatitis amongst the African natives, with the consequent development of cirrhosis and carcinoma (14, 22) *Berman* (4) on the other hand, according to an analysis of the diets of various African tribes, believes that cirrhosis is caused principally by a deficiency of proteins

In this study cirrhosis was found in 45 cases out of 104 patients with a distribution of 78 per cent in men and 22 per cent in women

The incidence of simultaneous cirrhosis in hepatocellular, cholangiocellular, and mixed carcinomas was observed to be 49, 33, and 18 per cent respectively

Epidemical hepatitis as a possible etiological factor for primary carcinoma of the liver could not be established from this investigation Information of icterus in the case histories was only found in 10 cases, of which 2 must be discarded because of simultaneous cholelithiasis No conclusion could be drawn from this small number, which, however, must be considered with the reservation to which anamnestic information is always subject, as negative answers are not explicitly noted There is also the possibility that some of the patients may not have noticed a mild jaundice, and that a reduced memory in this age group may influence the reliability of any information given

In an attempt to demonstrate a connection between epidemical hepatitis and cirrhosis, all 14,881 autopsies were investigated with reference to the incidence of cirrhosis which might reflect the increased frequency of hepatitis during and after the Second World War

For the two eleven-year periods this incidence, however, was practically unchanged, being 5 5 per cent and 5 9 per cent respectively

A year by year analysis showed the same constant proportion to the number of autopsies

The frequency of malignant degeneration in cirrhotic livers was found to be 3 3 per cent for the first eleven year period and 6 6 per cent

from an invasive gall bladder carcinoma If true then the entire group has been incorrectly diagnosed as primary carcinoma of the liver As, however, the incidence of gall bladder carcinoma, in relation to the total number of carcinomas, was observed to be exactly identical for the two eleven year periods, and as, according to *Kohn*, the frequency of gall bladder carcinoma has not increased, a possible conclusion may be that an error in diagnosis has been committed equally in the two periods, and subsequently has not influenced the observed rise in the number of primary liver carcinomas

The histology of these two types of carcinomas offers no support for the differential diagnosis Unfortunately it has only been in recent times that a systematic histological examination of sections, including both the liver tissue and the wall of the gall bladder, has been done to determine the origin of the tumour

From the macroscopic descriptions in 19 cases it was, however, quite obvious that the tumour originated from the bed of the gall bladder, and that the mucous membrane of the gall bladder was not involved

Histological examination in three cases confirmed that the tumour invaded the connective tissue of the bed of the gall bladder, but left the wall unaffected The gall bladder was embedded in the tumour of the liver in the remaining 7 cases with visible invasion of the mucous membrane in 3 cases

Metastases

Intrahepatic metastases occurred in 56 patients and extrahepatic secondary growths in 58 cases, of which 30 were multiple

The metastases were located as follows Lymph nodes, 30 cases lungs 9 cases, pleurae, 6 cases, other organs, 11 cases

Out of the total 104 cases only twelve cases were observed to be without any secondary tumours As a result the nihilistic point of view concerning therapy is considered fully justified, even though successful surgical removal of primary liver carcinoma has been reported with more than 5 year period of survival (13, 21, 23)

DISCUSSION AND CONCLUSION

The increased incidence of primary liver carcinoma from 0 38 per cent to 0 91 per cent in relation to the number of autopsies, and from 1 89 per cent to 3 29 per cent in relation to the number of carcinomas of other organs for two eleven year periods in this post mortem study, considerably exceeds the rise stated by other authors

The total number of hospital admissions for the periods shows an increase of 47 per cent, with an approximately identical mortality of 5 7 per cent and 5 8 per cent respectively

The autopsy rate has only indicated an insignificant rise from 80 per cent to 85 per cent

bile, and the intermediate liver parenchyme is often cirrhotic and indistinct

The typical nodular forms are often referred to as evidence of a multicentric development of carcinoma A slowly growing primary tumour invading a vessel in the highly vascularized liver may, however, cause a similar nodular appearance

As, according to literature, sufficient histological examinations regarding these problems apparently are not undertaken, the theory of a multicentric as against a unicentric origin of the liver carcinoma is still open to question

A detailed classification of the tumour localisation was made, the purpose of this work being originally to investigate the incidence of primary carcinoma of the liver in the region bordering the bed of the gall bladder

The following 6 groups were selected, in which the total number of tumours can be properly tabulated

Group 1 Characterized by a large solid tumour in the right lobe with multiple secondary growths in the rest of the liver Comprises 25 per cent (20 men and 6 women)

Group 2 A solid tumour in the left lobe In other respects like group 1 Comprises 15 per cent (12 men and 4 women)

Group 3 The nodular tumours Comprises 20 per cent (13 men and 8 women)

Group 4 Characterized by a situation in close relationship to the bed of the gall bladder Comprises 25 per cent (10 men and 16 women)

Group 5 Characterized by a central tumour without intrahepatic metastases Comprises 14 per cent (11 men and 4 women)

In accordance with literature a frequent localisation was demonstrated in the right lobe of the liver (2, 4, 17, 19), this prevalence being undoubtedly caused by the larger volume of the right lobe

An equally high percentage of tumours, however, was demonstrated (25 per cent) with an intimate relationship to the bed of the gall bladder As this localisation has not been reported in literature, a more detailed analysis of this group would be justified

Compared with the other groups, it showed a definite majority of women, which is even more remarkable considering the otherwise predominance of men Further a strikingly high frequency of simultaneous cholelithiasis was established, 54 per cent as against 26 per cent, as also a relative dominance of cholangiocellular carcinoma 50 per cent as against 34 per cent Finally simultaneous cirrhosis occurred in a considerably lower percentage, 27 per cent as against 47 per cent in the other groups

The characteristics of this group, all fitting the characteristics of gall bladder carcinomas, might imply that these tumours have originated

an increasing incidence of primary liver carcinoma in the older age-groups

The carcinomas occur more frequently in men, and the hepatocellular carcinoma is dominant histologically

The histological types are discussed and the histogenetic basis for the accepted classification in hepatocellular and cholangiocellular carcinomas is not found to be convincing

Simultaneous cirrhosis occurs in 43 per cent of the cases, but is demonstrated to be of no importance in the increased incidence of primary liver carcinoma No special relationship between cases of hepatitis and carcinoma of the liver can be established

A frequent localisation of tumours in close relationship to the bed of the gall bladder is discussed and the possibility of a development of carcinoma in the deep-seated crypts and ducts in the wall of the gall bladder is suggested as a cause for this particular localisation

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The increased number of admissions to this hospital in connection with the recent tendency to discharge hopeless cases, in order to obtain room for other patients more suited for therapy, would certainly cause a relative decrease in the number of carcinomas available for autopsy. The rapid fatal outcome of primary liver carcinoma means that almost all of these patients remain in the hospital, and thus this type of carcinoma will dominate the post mortem statistics.

The special department present in this hospital for the more chronic and hopeless cases would, however, reduce this influence, especially as the number of patients in this special department was found to have risen considerably in the second eleven-year period.

Although an exact analysis of all the circumstances affecting the incidence of primary carcinoma of the liver is not possible, the observed increase in this study is of such magnitude that it would be reasonable to conclude that primary carcinoma of the liver is a disease which is appearing with an increased frequency.

From the knowledge that hepatitis may develop into cirrhosis, which is precancerous, the recent hepatitis epidemics were thought to be responsible for this increased incidence of primary liver carcinoma.

This assumption, however, has not been verified. A history of jaundice was only reported in few cases, and the incidence of cirrhosis was found to be practically identical for the two eleven-year periods.

Finally the increase in the number of primary liver carcinomas was found in an almost equal degree in patients with cirrhosis as well as in patients without cirrhosis.

On the other hand the higher incidence of primary liver carcinoma in the older age-groups appears to be of significance, and it seems fairly reasonable to conclude that the prolonged life longivity alone is responsible for the increased incidence of primary carcinoma of the liver.

The relatively large group of carcinomas located in close relationship to the bed of the gall bladder is disputable, and the possibility of a primary gall bladder carcinoma invading the liver must be taken into consideration. In the majority of cases it is, however, evident that the mucous membrane of the gall bladder is not involved.

A malignant degeneration of the ectopic epithelium of Rokitansky-Aschoff's sinuses and Luschka's bile-ducts might theoretically contribute to this large group of carcinomas.

A histological examination of the topographical relations of these tumours may confirm such a theory and will be performed in the future in an attempt to clarify this possibility.

SUMMARY

In a post mortem study of 104 cases of primary carcinoma of the liver a significant increase in the incidence of this disease is demonstrated. This rise is probably connected with the prolonged life longivity with

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THE OCCURRENCE OF LIPOPEROXIDES IN HUMAN ADIPOSE TISSUES

By

J. GLAVIND, S. HARTMANN[1] and A. ZACHO

Received 3.1.61

The most important form of deterioration of fats and fat products is characterized by peroxide formation. Peroxide groups are formed on the unsaturated fatty acid components of the fats through the action of atmospheric oxygen by a free radical mechanism. The peroxides initially formed (lipoperoxides) are highly reactive and may undergo chain scission, polymerization and other reactions whereby a great variety of secondary oxidation products are formed.

[illegible]

vitamin E in the animal organism as an antioxydant, preventing the formation of lipoperoxides, is now well established.

One aspect of the antioxydant function of vitamin E has been studied in several laboratories, *viz.*, differences between tissues and subcellular structures from normal and vitamin E-deficient animals with respect to postmortem peroxide formation under certain circumstances. The studies were made possible by the introduction of the thiobarbituric acid reaction (9) which, although not given by the lipoperoxides proper, but by certain secondary reaction products, may give interesting information about peroxide formation.

Although most experimental studies using the thiobarbituric acid reaction have centered on postmortem peroxide formation, also its use for the demonstration of *in vivo* peroxides in vitamin E-deficient animals has been attempted (8). *Dubouloz & Dumas* (3), using quite a different method, demonstrated the presence of true peroxides in experimental inflammation.

The occurrence of lipoperoxides in human pathology has been demonstrated in adipose tissue by peripheral venous diseases (7), and in the atherosclerotic aorta (4).

As to normal tissue *Dubouloz et al.* (2) by their own method have

[1] Deceased May 1959.

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anaerobically A series of extracts of adipose tissues were examined by both methods Hereby a factor of 0 4 was found by which the results obtained with the indophenol method must be multiplied to be compared with those obtained with the thiocyanate method Evidently, since the discrepancy between the two methods is due to the interference of oxygen the use of a conversion factor will permit only a rough comparison between the results obtained by them Since our determinations were carried out in a very uniform way, the use of a conversion factor was considered permissible

The anaerobic thiocyanate method is believed to estimate the true amounts of lipoperoxide, and all the results are given as microequivalents of lipoperoxide per gram fat

DISCUSSION

The results presented in the figure show that while in many cases no peroxide could be demonstrated in the lipids of human adipose tissues, in other cases a small, but distinctive value was found A small value was also found in two lipomas

By the technique used it seems most probable that the peroxides found were present *in vivo* From trials with adipose tissues from calves it appears that very little postmortem formation of peroxides occurs when the tissues are stored in the cold for 1–2 days

An examination of the case papers did not reveal any correlation between diagnosis etc , and peroxide content Especially, no positive or negative correlation was found between the occurrence of cancer and of peroxide The material mostly consisted of older and middle aged cases, no young subject was included

The only antioxidants known to be physiologically active in the animal organism are the tocopherols Obviously, since very little or no peroxide is found in the fat tissues, also other mechanisms for the prevention of peroxide formation must exist in the organism which may act in context with the processes of continuous mobilization and replenishment of the lipids From our results it appears that the mechanisms do not always completely suffice According to what is known from animal experiments and from human patholo[illegible] differences in the case material or between local sites of the tissue samples regarding such factors may explain the small differences as to the peroxide contents observed in our study

SUMMARY

A material of 86 samples of human adipose, mainly subcutaneous tissue and two lipomas, taken in connexion with operations of various type was examined for peroxide content Sometimes no peroxide and

found minute amounts of lipoperoxides in adipose tissues of rats and dogs (about 0 1–0 2 micromol per g) We likewise found small quantities of peroxides in pigs' adipose tissue, whereas none could be demonstrated in calves and cows Minute amounts were also found in freshly drawn cow's milk (unpublished results) However, as far as we know, a study on the occurrence of lipoperoxides in a larger material of normal tissues has never been carried out

In the following we shall report our studies on a material of human adipose tissue taken in connection with operations and examined as quickly as possible in order to eliminate postmortem peroxide formation

MATERIAL AND METHODS

The tissue samples were removed in connexion with operations of various types A great deal of the operations were for cancer Only tissues consisting largely of lipids were examined In most cases a piece of adipose tissue in other cases of omentum was taken Finally, two lipomas were included in the material

Immediately after removal the tissue was placed in a cooled glass placed for a short time in an ice box packed in insulating material and sent to the laboratory In most cases the analysis could be carried out in about two hours after the operation

The fat tissue was disintegrated in a mortar with anhydrous sodium sulphate extracted with chloroform filtered and the extract evaporated *in vacuo* at low temperatures The fat was measured with a pipette, as a rule 1 ml was used in the determination Corrections were made for the color of the extract and for a reagent blank

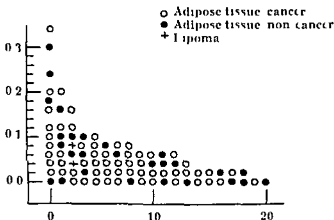


Fig 1
Peroxide content of human fat
Abscissae Number of cases
Ordinates Peroxide microequivalents per gram

The determinations were carried out through a longer period during which it was established that the method originally used, the so-called indophenol method (6) gave too high results, due to the fact that atmospheric oxygen was not excluded The last part of the determinations were made with the thiocyanate method (5) which was carried out

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GENERALIZED CYTOMEGALIC INCLUSION DISEASE IN A NEWBORN INFANT

By

ODD GARDBORG and ODD F HANSSEN

Received 26 I 61

Inclusion body disease (I B D cytomegalia infantum salivary gland virus disease) is recognized by the demonstration in various viscera of typical giant cells with characteristic intranuclear and cytoplasmatic inclusion bodies. These cells were first described by *Jesionek & Kiolemenoglou* in 1904 (6) but were observed by *Ribbert* as early as 1881 in the kidney of a newborn infant and in the parotid glands of 2 older infants (12).

Numerous cases of I B D have later been described from different parts of the world. The disease has earlier been of interest mostly to pathologists. Since inclusion bearing cells are often desquamated into the renal tubules *Wyatt et al* (20) suggested that it may be possible to make a diagnosis ante mortem by cytological studies of the urine and in 1951 *Fetterman* demonstrated such cells in the urine of a premature infant who died at four days of age of generalized inclusion disease (4). The diagnosis was also established intra vitam by *Mercer et al* in 1953 (11). In Scandinavia *Ahvenainen* has described 5 cases from Finland in 1951 (1) and the first case diagnosed in Sweden has recently been published by *Bjorklund & Wiebert* (3).

by

the found is a result of a viral infection. In 1956 filtrable agents capable of producing the typical incl ons

et al also isolated a virus from liver tissue and urine of patients with I B D and demonstrated antibodies in the serum not only from patients with the disease but also frequently from healthy individuals (19).

to

gn is most frequently seen in prematures. The clinical picture is dominated by jaundice, a thrombocytopenic pur

sometimes very small values (0–0 3 μequiv per g fat) were found No correlation between peroxide content and diagnosis or other clinical observations could be demonstrated

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The blood picture showed an anemia with reticulocytosis leucocytosis and thrombocytopenia The blood group was AB, Rh positive (the blood group of the mother A Rh positive) The Coombs' test was negative The results of the most important laboratory tests are given in Table 1

The spinal fluid was clear yellow, contained 3/3 cells and showed negative protein

[illegible]

nitis on the left side

On the basis of these findings a tentative diagnosis of I B D was made, but cytologic examination of urine sediment and spinal fluid failed to reveal any inclusion-bearing cells Since a septicemia could not be excluded the patient was treated with

[illegible]

in the hospital

Autopsy Findings

Gross examination revealed a dystrophic and icteric child, weighing 2600 g The lungs showed a patchy dark consolidation Microscopical examination revealed a purulent bronchitis and bronchopneumoonia There were no effusions in the pleural or pericardial cavities The peritoneal cavity contained about 200 ml of yellowish fluid

The heart appeared normal

The liver, weighing 130 g, showed a granular surface Microscopically, a slight periportal fibrosis was found with evidence of bile duct proliferation The centrolobular liver cells were atrophic and pigmented and the bile capillaries contained bile thrombi The gall bladder and the bile ducts appeared normal

The spleen, weight 63 g, was enlarged, and microscopic examination revealed evidence of hematopoiesis

In the kidneys the cortex appeared somewhat blurred Microscopically, small scattered subcapsular areas were found in which the tubules were atrophic and the interstitium was infiltrated with lymphocytes

Examination of the brain showed an old hemorrhage in the area of vena terminalis with accompanying gliosis in the surrounding gray and white matter There was evidence of retarded development of the cortex and degenerative changes were found in the cranial nerve nuclei (Dr Yngol Loken)

The diagnostic features of inclusion body disease were the presence of cytomegaly with inclusion bodies These were [illegible] the ducts of [illegible] hi, the lungs, [illegible] in the renal [illegible] cells including the nuclei were considerably

pura, hepato-splenomegaly, hemolytic anemia with reticulocytosis, and leucocytosis with a shift to the left of the neutrophils Signs of cerebral damage are often present in the form of intracranial calcifications, hydrocephalus, microcephalus and convulsive seizures (5, 10) Typical inclusion-bearing cells have been demonstrated in placenta (8) The clinical picture can be very similar to erythroblastosis foetalis, however, no blood incompatibility between mother and child can be demonstrated and the Coombs test is negative It is also necessary to exclude toxoplasmosis, lues congenita, sepsis neonatorum and other diseases which can cause jaundice and thrombocytopenia in the newborn (10)

The clinical diagnosis must be based on the demonstration of the typical inclusion-bearing giant cells in the urine, gastric washings or spinal fluid (4, 15) A parotid biopsy may also be useful, as it seems likely that demonstration of parotis cytomegalia in typical cases of congenital I B D is sufficient for the diagnosis (18) If possible, isolation of the virus from the urine and demonstration of neutralizing antibodies in the blood should be attempted There seems to be a tendency for infected individuals to continue to excrete the virus in the urine or saliva (7, 14, 19)

CASE REPORT

A 2½ month old male infant I W born on January 29 1959 was admitted to the Children s Hospital Rikshospitalet for jaundice Both parents were healthy and pregnancy and delivery had been normal Birth was said to have taken place at term but the birth weight was only 2340 g

The patient appeared weak after birth and was treated with oxygen the two first days of life Jaundice and numerous petechiae were noted during the first 24 hours of life Hemolytic disease of the newborn was suspected but no blood incompatibility could be demonstrated and the Coombs test was negative The following days the patient developed a pronounced jaundice and a markedly enlarged liver and spleen

The patient was breast fed the first 2 weeks of life later he was placed on a cowmilk regimen He remained icteric with gray stools and showed a constant failure to thrive At the age of 4 weeks he was treated for one week with cortone (25 mg daily) and terramycine without any effect

On admission to the Children s Hospital the patient was in a poor condition icteric and with a pronounced dystrophic appearance His weight was 2420 g rectal temperature 37 1° C The clinical examination revealed a distended abdomen and a markedly enlarged liver and spleen After a few days he also developed abundant petechiae

TABLE 1

Age in weeks	Hemoglobin g 100 ml	Erythrocytes mill cmm	Leucocytes	Reticulocytes ‰	Thrombocytes	Bilirubin mg 100 ml	Prothrombin %
11	9 3	3 40	57300	152	41700	5 8	70
12	8 5	3 36	32600	42	36900	5 4	64
13	8 0	2 76	40300		82800	5 4	
14	6 2	2 36	25300	46			52
15	4 2	1 35	31600	60			

The blood picture showed an anemia with reticulocytosis, leucocytosis and thrombocytopenia. The blood group was AB Rh positive (the blood group of the mother A Rh positive). The Coombs test was negative. The results of the most important laboratory tests are given in Table 1.
The spinal fluid was clear yellow, contained 3/3 cells and showed negative protein reactions. Blood urea was 28 mg/100 ml, thymol 1.03. The electrophoretogram revealed the following levels: total proteins 6.4 g/100 ml, albumin 3.2 g/ml, globulin 3.2 g/100 ml, alpha₁ globulin 0.3 g/ml, alpha₂ globulin 0.4 g/100 ml, beta globu

penicillin and chloromycetin in massive doses.
The patient remained in a poor condition the whole time. He was placed on a cow milk regimen. The feeding was difficult, the patient vomited daily and failed to gain in weight. After 3 weeks his condition became worse with increasing anemia (Table 1). Blood transfusions gave no result and death ensued after 4 weeks stay in the hospital.

Autopsy Findings

Gross examination revealed a dystrophic and icteric child weighing 2600 g. The lungs showed a patchy dark consolidation. Microscopical examination revealed a purulent bronchitis and bronchopneumoonia. There were no effusions in the pleural or pericardial cavities. The peritoneal cavity contained about 200 ml of yellowish fluid.

The heart appeared normal.

The liver weighing 130 g showed a granular surface. Microscopically a slight periportal fibrosis was found with evidence of bile duct proliferation. The centrolobular liver cells were atrophic and pigmented and the bile capillaries contained bile thrombi. The gall bladder and the bile ducts appeared normal.

The spleen weight 63 g was enlarged and microscopic examination revealed evidence of hematopoiesis.

In the kidneys the cortex appeared somewhat blurred. Microscopically small scattered subcapsular areas were found in which the tubules were atrophic and the interstitium was infiltrated with lymphocytes.

Examination of the brain showed an old hemorrhage in the area of vena terminalis with accompanying gliosis in the surrounding gray and white matter. There was evidence of retarded development of the cortex and degenerative changes were found in the cranial nerve nuclei (Dr *Inger Laken*).

The diagnostic features of inclusion body disease were the presence of cytomegaly with inclusion bodies. These were found in the ducts of the submandibular gland, the mucous glands of the bronchi, the lungs, in the exocrine and endocrine tissue of the pancreas and in the renal tubules. The affected cells including the nuclei were considerably

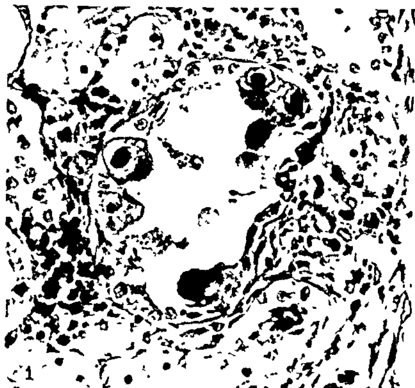


Fig 1

Fig 1 shows a renal tubule with considerably enlarged epithelial cells bulging into the lumen The nuclei and the cytoplasm contain basophilic inclusion bodies HE, × 440

enlarged (Figs 1, 2 and 3) The nucleus contained a dense basophilic inclusion body and the nuclear substance was reduced to a thin ring surrounding the inclusion body After staining with Feulgen, the inclusion bodies appeared red as did the nuclear chromatine (Figs 2 and 3), and granular masses with the same staining properties were also evident in the cytoplasm of the cells (Figs 2)

COMMENT

The patient described above is the first cases of congenital generalized inclusion body disease diagnosed in Norway Although the disease seems to occur with a low frequency, it is reasonable to believe that the diagnosis may be established more frequently when the physicians become more familar with the clinical picture

In recent time several cases of I B D have been diagnosed intra vitam, and some of the reported cases have survived after treatment with corticoids *Margileth* (9) and *Birdsong et al* (2) have each described one case apparently cured after treatment with cortisone and prednisone respectively, however, in the first case sequelae of cerebral damage persisted The case published by *Van Gelderen* (18) was treated with prednisone for three months, and by a follow up at the age of 13 months,

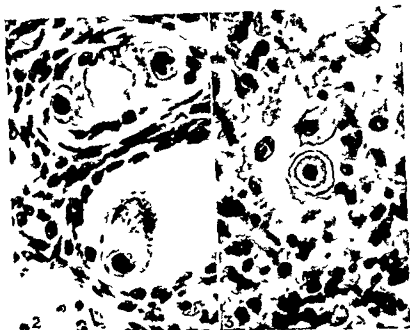


Fig 2

Fig 2 demonstrates Feulgen positive inclusions contained within the nuclei and the cytoplasma of affected epithelial cells of renal tubles Feulgen × 680

Fig 3

Fig 3 shows an intranuclear inclusion of a macrophage within the lung Feulgen × 680

this child was found completely healthy *Jelong et al* (8) have described a similar case treated with exchange transfusions and Cortancyl and this child was found normal in every respect at the age of 2 years and 4 months

At the present time it is of course difficult to judge to what extent corticoid treatment can improve the prognosis of I B D This is especially so as cases have been reported which have survived without any corticoid treatment However, because of the few promising results so far obtained in a disease which has usually been fatal it seems justified to try corticoid treatment in cases of neonatal I B D and it is of course of utmost importance to establish the diagnosis and start treatment as soon as possible after birth

SUMMARY

A case of inclusion body disease in a newborn male infant is described On the basis of a typical clinical picture a tentative diagnosis of I B D was made intra vitam but cytologic examination of urine sediment and spinal fluid failed to reveal any inclusion bearing cells

The patient died 3½ months old By autopsy inclusion-bearing giant cells were demonstrated in the submandibular glands, the lungs, pancreas and kidneys

The patient is the first case of congenital inclusion body disease diagnosed in Norway

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GLUCOSE CATABOLISM IN NEISSERIA MENINGITIDIS

1. *Glucose Oxidation and Intermediate Reactions of the Embden Meyerhof Pathway*

By

K. Jyssum, Bjørg Borchgrevink and Sidsel Jyssum

Received 21 xii 60

A phosphorylase from N. meningitidis catalyses the reversible phosphorylation of maltose plus inorganic phosphate to β-D-glucose-1-phosphate and D-glucose. This phosphate of glucose is accumulated in the medium while the glucose moiety from the original maltose molecule is further metabolized (*Fitting & Doudoroff* 1952). The pathways, however, by which glucose is broken down are little known. In this laboratory it has previously been found that meningococci may be adapted to growth with maltose or glucose as their sole source of carbon and energy (*Jyssum* 1959). The "secondary" pathways of glucose catabolism, *i.e.* those for which the immediate degradation products of glucose such as pyruvate or acetate are serving as the substrate, have been the subject of a previous paper (*Jyssum* 1960). The only reaction of the classical tricarboxylic acid cycle to which no corresponding activity could be demonstrated was the DPN dependent malic dehydrogenase. Meningococci also appeared to lack the enzymes aspartase and isocitratase.

The studies reported in the present publications were undertaken to elucidate the "primary" pathway from glucose to pyruvate and acetate in "resting cells" of N. meningitidis adapted to growth with glucose as their sole source of energy and carbon, and all nitrogen provided as the ammonium ion.

MATERIALS AND METHODS

Preparation of suspensions and extracts. The methods generally used in order to obtain material for the present studies followed those previously described (*Jyssum* 1959, *Jyssum & Borchgrevink* 1960).

[illegible] ion were measured at 37° [illegible] (1958). Corrections were [illegible] spectrophotometer was used in spectrophotometric analyses.

Analytical procedures. Reduction and oxidation of pyridin nucleotide coenzymes were followed at 340 mμ. Protein was measured by the method of [illegible] (1941), and nitrogen by a micro [illegible] Summerson (*Colorimetric* [illegible] rker & [illegible] ne ex[illegible]traction method (*Lof* [illegible] 1958).

were used for the estimation of lactate and pyruvate respectively. Pyruvate was also determined enzymatically with lactic dehydrogenase. In the latter method the reaction was stopped and the reaction mixture deproteinized by the addition of equal volume of 6 per cent (w/v) perchloric acid. To the acid supernatant K_2HPO_4 was added until pH 6.5, and after removal of the sediment a suitable aliquot was analysed with LDH and DPNH. Acetate was estimated by titration of the total steam volatile fraction in a Markham still. The concentration of acetate was obtained by subtracting the formate content after determining the latter manometrically with mercuric acetate (*Pickett* 1943). Triosephosphate was determined as alkali labile phosphate which was analysed by the method of Fiske & Subbarow (*Colowick & Kaplan* 1955–1957), or enzymatically by means of triosephosphate isomerase (TIM) plus α glycerophosphate dehydrogenase (GDH). In the enzymatic assay the reaction mixture was deproteinized and neutralized as described for the pyruvate assay. A suitable aliquot was analysed in the conventional way with TIM, GDH and DPNH.

The chromatographic procedures were generally the same which have previously been described (*Jyssum* 1960). Phosphorylated compounds were analysed by descending chromatography *in a system with 500 g phenol dissolved in 13 ml 90 per cent* HCOOH plus 167 ml H_2O (*Kornberg* 1958), in butanol propionic acid water (*Calvin & Benson* 1949) or in ethanol methanol water = 9 9 2 (*Norris & Campbell* 1949). The compounds were detected on the paper by the method of Hanes & Isherwood. Sugars and other reducing substances were detected with a saturated solution of aniline oxalate or by spraying with ammoniacal silver nitrate and exposure to UV light (*Block, Durrum & Zweig* 1958).

Chemicals. TPN, DPN, TPNH, DPNH and 6 phosphogluconate were purchased from Sigma Chemical Co. α glycerophosphate dehydrogenase, triosephosphate isomerase, glyceraldehyde dehydrogenase and lactic dehydrogenase were the products of C. F. Boehringer & Soehne GmbH, Mannheim, Germany. Other chemicals were obtained from the firms previously stated (*Jyssum & Borchgrevink* 1960) and used without further purification.

Symbols. In general the symbols and abbreviations from the publication Methods in Enzymology is followed (*Colowick & Kaplan* 1955 1957).

RESULTS

Oxidation of Glycolytic Intermediates and Related Compounds

Suspensions of meningococci actively catalyse the oxidation of maltose, glucose, glucose-6-phosphate, fructose-6-phosphate, fructose-1,6 diphosphate and triosephosphate when oxygen is used as the final electron acceptor in the way it has been shown in Fig 1 and Fig 2. Oxidation is also found with 6-phosphogluconate and ribose-5 phosphate as substrates.

Various nonphosphorylated compounds were also tested. Glycerol as well as both trioses were oxidized even if the oxidation rates, specially of the latter were very slow. δ gluconolactone, ribose or ribulose as well as seduheptulose were not oxidized, neither was fructose. Respiratory quotients for substrate mediated oxidations were measured by gas exchanges over various periods in the Warburg indirect and direct techniques. When allowance was made for the endogenous activity in calculating both methods gave as results quotients with values close to one. A typical experiment with glucose as the substrate gave the following quotients in the indirect technique:

After 30 minutes RQ = 0.80
After 50 minutes RQ = 1.03
After 95 minutes RQ = 1.01

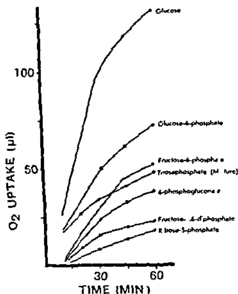


Fig 1

Oxidat on of glucose and phosphorylated compounds by suspensions of N meningitidis Warburg flasks contained approximately 0 5 g cells (wet weight) 180 µmoles phosphate buffer pH 7 4 5 µmoles substrate which was added from a sidearm at zero time Total volume of fluid was 3 2 ml The experiments were run at 37° Endogenous uptake is subtracted

Also by oxidations of phosphorylated intermediates the gas exchange experiments resulted in quotients with values close to one

Comparative studies showed that the presence of extracellular inorganic phosphate was not a limiting factor in the oxidation of either non phosphorylated or phosphorylated compounds On the contrary inorganic phosphate appeared to depress the over all oxidation of glucose In the presence of extracellular phosphate the oxidation of glucose presented a two phase curve At first a rapid oxidation took place until an approximate consumption of 2 micromoles oxygen per micromole glucose After this period the oxidation proceeded at a slower rate until between 3 and 4 micromoles oxygen had been absorbed per micromole glucose added With tris buffer and no added phosphate the absorption of approximately 4 micromoles oxygen per micromole substrate took place with the same rapid rate These features have been illustrated by the results recorded in fig 2 In many experiments the level of 4 micromoles oxygen per micromole glucose has been reached It has not been possible, however to demonstrate a further uptake of oxygen

Since the values for oxygen consumption did not correspond to the complete oxidation of the substrate added the reaction mixtures were analysed for reaction products other than CO From the data presented

were used for the estimation of lactate and pyruvate, respectively. Pyruvate was also *determined enzymatically with lactic dehydrogenase. In the latter method the* reaction was stopped and the reaction mixture deproteinized by the addition of equal volume of 6 per cent (w/v) perchloric acid. To the acid supernatant K_2HPO_4 was added until pH 6.5, and after removal of the sediment a suitable aliquot was analysed with LDH and DPNH. Acetate was estimated by titration of the total steam volatile fraction in a Markham still. The concentration of acetate was obtained by subtracting the formate content after determining the latter manometrically with mercuric acetate (*Pickett* 1943). Triosephosphate was determined as alkali labile phosphate which was analysed by the method of Fiske & SubbaRow (*Colowick & Kaplan* 1955–1957), or enzymatically by means of triosephosphate isomerase (TIM) plus α glycerophosphate dehydrogenase (GDH). In the enzymatic assay the reaction mixture was deproteinized and neutralized as described for the pyruvate assay. A suitable aliquot was analysed in the conventional way with TIM, GDH and DPNH.

The chromatographic procedures were generally the same which have previously been described (*Jyssum* 1960). Phosphorylated compounds were analysed by descending chromatography in a system with 500 g phenol dissolved in 13 ml 90 per cent HCOOH plus 167 ml H_2O (*Kornberg* 1958), in butanol propionic acid water (*Calvin & Benson* 1949), or in ethanol methanol water = 9 9 2 (*Norris & Campbell* 1949). The compounds were detected on the paper by the method of Hanes & Isherwood. *Sugars and other reducing substances were detected with a saturated solution* of aniline oxalate or by spraying with ammoniacal silver nitrate and exposure to U.V. light (*Block, Durrum & Zweig* 1958).

Chemicals. TPN, DPN, TPNH, DPNH and 6 phosphogluconate were purchased from Sigma Chemical Co. α glycerophosphate dehydrogenase, triosephosphate isomerase, glyceraldehyde dehydrogenase and lactic dehydrogenase were the products of C. F. Boehringer & Soehne GmbH Mannheim Germany. Other chemicals were obtained from the firms previously stated (*Jyssum & Borchgrevink* 1960) and used without further purification.

Symbols. In general the symbols and abbreviations from the publication Methods in Enzymology is followed (*Colowick & Kaplan* 1955 1957).

RESULTS

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Suspensions of meningococci actively catalyse the oxidation of maltose, glucose, glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate and triosephosphate when oxygen is used as the final electron acceptor in the way it has been shown in Fig. 1 and Fig. 2. Oxidation is also found with 6-phosphogluconate and ribose-5-phosphate as substrates.

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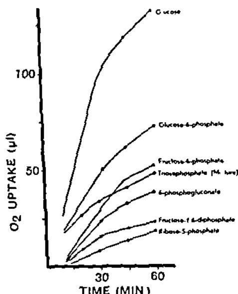


Fig 1

Oxidation of glucose and phosphorylated compounds by suspensions of N. meningitidis. Warburg flasks contained approximately 0.5 g cells (wet weight), 180 µmoles phosphate buffer pH 7.4, 5 µmoles substrate which was added from a sidearm at zero time. Total volume of fluid was 3.2 ml. The experiments were run at 37°. Endogenous uptake is subtracted.

Also by oxidations of phosphorylated intermediates the gas exchange experiments resulted in quotients with values close to one.

Comparative studies showed that the presence of extracellular inorganic phosphate was not a limiting factor in the oxidation of either non phosphorylated or phosphorylated compounds. On the contrary, inorganic phosphate appeared to depress the over all oxidation of glucose. In the presence of extracellular phosphate the oxidation of glucose presented a two phase curve. At first a rapid oxidation took place until an approximate consumption of 2 micromoles oxygen per micromole glucose. After this period the oxidation proceeded at a slower rate until between 3 and 4 micromoles oxygen had been absorbed per micromole glucose added. With tris buffer and no added phosphate the absorption of approximately 4 micromoles oxygen per micromole substrate took place with the same, rapid rate. These features have been illustrated by the results recorded in fig. 2. In many experiments the level of 4 micromoles oxygen per micromole glucose has been reached. It has not been possible, however, to demonstrate a further uptake of oxygen.

Since the values for oxygen consumption did not correspond to the complete oxidation of the substrate added, the reaction mixtures were analysed for reaction products other than CO_2. From the data presented

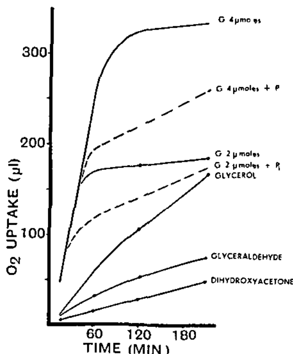


Fig 2

Oxidation of glucose and trioses by suspensions of N meningitidis Effect of inorganic phosphate Warburg flasks contained approximately 0 5 g cells (wet weight) 180 µmoles phosphate buffer or 180 µmoles tris buffer The substrate was tipped in from a sidearm at zero time Total volume of fluid was 3 2 ml The experiments were run at 37° Endogenous uptake is subtracted The following substances gave no uptake of oxygen δ gluconolactone, fructose ribose ribulose and seduheptulose

in Table 1 it may be seen that the main reaction product besides CO_2 is acetic acid No accumulation took place of lactic, pyruvic or formic acid The products recovered account for at least 95 per cent of the carbon metabolized during those experiments where tris buffer was used With inorganic phosphate present the oxidation did not proceed to the same level, and the carbon recovery among the products analysed was less

Identification of Intermediate Reactions of the Embden Meyerhof Pathway of Glycolysis

Glucose-6-phosphate dehydrogenase The presence of an activity corresponding to a G-6-P dehydrogenase was demonstrated by the direct reduction of pyridin nucleotide coenzymes in the way it has been illustrated in Fig 3 It is seen that both TPN and DPN were active in this reaction In view of the fact that crude extracts were used it may be suggested that the DPN reduction was caused by a transhydrogenase In previous studies, however, no evidence of such an enzyme activity has been demonstrated A similar activity of DPN with crude extracts was not found in other, strictly TPN dependent reactions such as the

TABLE 1

Products of Glucose Oxidation by Neisseria meningitidis

Products analysed	Results (µmoles/µmole substrate) Tris buffer	Phosphate buffer
CO_2	3.84	2.80
Acetic acid	1.01	1.18
Formic acid		
Lactic acid		
Pyruvic acid		
O_2 uptake	3.94	3.03
RQ	0.97	0.92
Carbon recovery per cent	97.6	86.0

Each reaction vessel contained 4 µmoles substrate, 100 µmoles tris or phosphate buffer pH 7.4, 1 ml of freshly harvested and washed *N. meningitidis* cells. Temperature 37° and aerobic conditions. The experiment with tris buffer was run for 4 hours and that with phosphate buffer for 6 hours, in both instances until no more oxygen was assimilated. CO_2 was determined by the indirect technique.

isocitric dehydrogenase (*Jyssum* 1960). Optimal rates for the G-6-P dehydrogenase activities were found between pH 8 and pH 8.5.

With crude concentrated extracts considerable reoxidation of DPNH occurred while TPNH was not affected. In agreement with previous findings the DPNH oxidation could be inhibited by the addition of cyanide (*Jyssum & Borchgrevink* 1960). The G-6-P dehydrogenase activities were also compared in extracts from "wild type" meningococci and from meningococci adapted to growth on a minimal medium

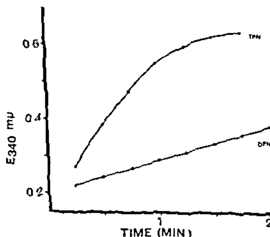


Fig. 3

Glucose-6-phosphate dehydrogenase activity in cell free extracts from *N. meningitidis*. The reaction mixture contained 100 µmoles barbital buffer pH 8, 0.25 µmoles TPN or DPN, 10 µmoles G-6-P as potassium salt, 0.5 ml *meningococcus* extract dilution.

with the ammonium ion as the sole source of nitrogen and glucose of energy and carbon It appears from several data compiled in Table 2 that no significant changes in the various activities may be demonstrated after the adaption The relationship between the activities of DPN and TPN in crude extracts could constantly be characterized by a quotient close to 0 4 in these dehydrogenations

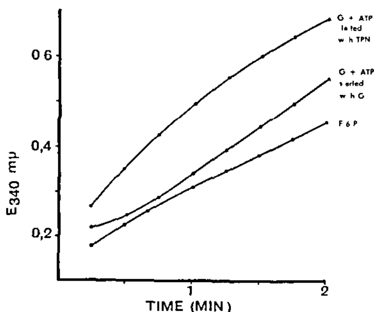


Fig 4

Glucokinase and phosphofructoisomerase activity in cell free extracts from N meningitidis *Experiments with glucose (G) as substrate* The cuvette contained 100 μmoles barbital buffer pH 8 5 μmoles $MgCl_2$ 0 25 μmoles TPN 2 μmoles ATP, 20 μmoles glucose, 0 5 ml meningococcus cell free extract dilution The reaction was started at zero time as marked on the curves after a preincubation of the rest of *the mixture for 5 minutes Experiments with F 6 P as substrate The mixture con*tained 100 μmoles barbital buffer pH 8, 0 25 μmoles TPN 20 μmoles F 6 P 0 5 ml meningococcus cell free extract dilution When tested with the same buffers and extracts the following systems gave no reaction ATP + TPN, Glukose + TPN Fructose + TPN Fructose + TPN + ATP

Glucokinase The existence of a glucokinase in cell-free extracts was *first demonstrated with the G-6 P dehydrogenase as indicator system* as shown in Fig 4 A comparison of the rate of TPN reduction with that of the direct assay with G-6 P as substrate, points out the kinase is the rate limiting factor in these extracts from N meningitidis This may also be read from the curves of Fig 4 It is seen that a preincubation of glucose plus ATP increases the initial rate of TPN reduction considerably when compared with the experiment where the reaction was started by the addition of the substrate, *i e* glucose With the kinase as the rate limiting factor the present type of assay may be assumed to give a fair means of comparison between the glucokinase activities of various meningococcal extracts

After adaption of the meningococci to growth with glucose as the source of energy and carbon extracts evinced a substantially increased hexokinase activity This may be read from the data presented in Table 2

TABLE 2

Activities Corresponding to Various Glycolytic Enzymes in Extracts from Neisseria meningitidis

Source of extract	Extract no	Activities E_{340} 10³ min 0 1 mg N			
		G-6-P dehydrogenase		Phosphofructo isomerase	Hexokinase
		TPN	DPN	TPN	TPN
M6 wild type	h	208 5	–	140 6	57 7
–	i	266 7	–	140 0	51.3
M6 adapted"	XIV	217 1	–	142 9	91 4
–	XV	282 7	–	197 3	94 5
	XVI	235 7	–	160 7	97 7
M6 wild type"	n	268 8	108 8	–	–
–	p	256 7	116 7		–
M6 "adapted"	XXIII*	173 2	74 2	–	–
–	XXXIV	273 4	111 6	–	–

* This extract was prepared from old cells which had been preserved in the frozen state

TABLE 3

Aldolase Activity in Cell free Extracts from Neisseria meningitidis

Extract no	Extract amount ml	pH	Inhibiting factor tested		Alkali labile phosphorus µmoles
1	0 2	8 4	None		1 32
	0 4	–	–		2 27
	0 6	–	–		3.37
2	0 2		–		1 21
–	–	–	$ZnSO_4$	20 µmoles	1.35
–	–	–	–	2 –	1 35
–	–	–	$CuSO_4$	2 –	0 13
–	–	–	–	0 5 –	0 22
–	–	–	CH_2ICOOH	2 6 –	1.32
–	–		Cysteine	12 5	1.35
–	–	–	Enz frozen for 24 h		1 17
–	–	–	– – – 96 h		0 90
–	–	8 0	None		1 53
3		8 4	–		1 57
	–				1 50
–		8 0			1 80
	–	–	–		1 84

The reaction mixture contained 12 5 µmoles F 1 6 P, 100 µmoles tris buffer, 55 µmoles hydrazine sulphate at desired pH, cell free extract dilution Total volume of the system was 2 5 ml The reactions were run for 15 minutes at 37° and stopped by the addition of TCA

with the ammonium ion as the sole source of nitrogen and glucose of energy and carbon It appears from several data compiled in Table 2 that no significant changes in the various activities may be demonstrated after the adaption The relationship between the activities of DPN and TPN in crude extracts could constantly be characterized by a quotient close to 0 4 in these dehydrogenations

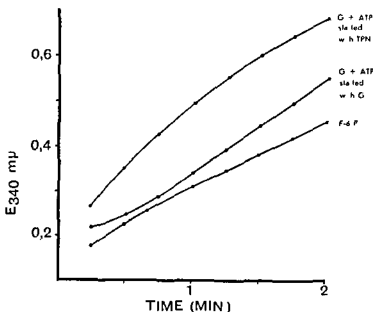


Fig 4

Glucokinase and phosphofructoisomerase activity in cell free extracts from N *meningitidis* *Experiments with glucose (G) as substrate* The cuvette contained 100 μmoles barbital buffer pH 8, 5 μmoles $MgCl_2$, 0 25 μmoles TPN, 2 μmoles ATP, 20 μmoles glucose, 0 5 ml meningococcus cell free extract dilution The reaction was started at zero time as marked on the curves after a preincubation of the rest of the mixture for 5 minutes *Experiments with F 6 P as substrate* The mixture contained 100 μmoles barbital buffer pH 8, 0 25 μmoles TPN, 20 μmoles F-6 P 0 5 ml meningococcus cell free extract dilution When tested with the same buffers and extracts the following systems gave no reaction ATP + TPN, Glukose + TPN Fructose + TPN, Fructose + TPN + ATP

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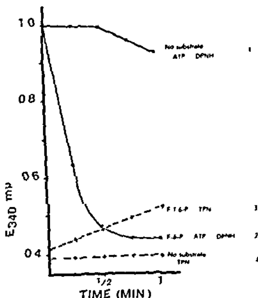


Fig 6

Fructose 6 phosphate kinase and fructose 1 6 diphosphate phosphatase activities in cell free extracts from N meningitidis *Curves 1 and 2* Meningococcus extract dilution 0 5 ml was incubated with 100 μmoles barbital buffer pH 8 5 μmoles $MgCl_2$ 2 μmoles ATP 0 01 ml GDH/TIM and 20 μmoles F 1 6 P for 30 minutes at 37° The reaction proper was started at zero time by the addition of 0 5 μmoles DPNH *Curves 3 and 4* Meningococcus extract dilution 0 5 ml was incubated with 100 μmoles barbital buffer pH 8 5 μmoles $MgCl_2$ and 20 μmoles F 1 6 P for 20 minutes at 37° The reaction recorded was started at zero time by the addition of 0 25 μmoles TPN

shown in Table 3 The aldolase activity was also studied in a system where the oxidation of DPNH by α-glycerophosphate dehydrogenase was used as the indicator system for the formation of dihydroxyacetonephosphate Excess of triosephosphate isomerase was added during these experiments The aldolase effect in this system appears from the data presented in Fig 5

Experiments were also performed in which the system with α glycerophosphate dehydrogenase and triosephosphate isomerase was used for the analysis of triosephosphate from reaction mixtures where protein had been precipitated in the way it has been described under methods

The DPNH oxidase effect of crude extracts which is apparent from the data of Fig 5 could be completely inhibited by the addition of 1 to 2 μmoles KCN The carbonyl trapping activity of cyanide in these concentrations did not significantly influence the reaction as performed An orientation concerning the influence of metal ions on the aldolase

[illegible] The activating effect of inorganic phosphate which appears from the experiment re-

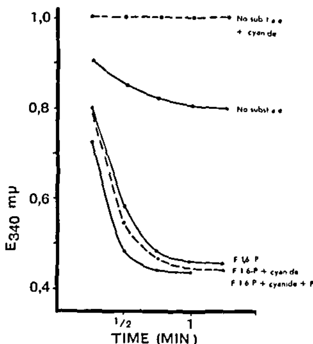


Fig 5

Enzymatic assay of aldolase activity in cell free extracts from N meningitidis A mixture of 0 5 ml extract dilution 100 µmoles barbital buffer pH 8 10 µmoles nicotinamide and 3 2 µmoles KCN was incubated at 37° for 7 minutes Then 0 01 ml GDH/TIM plus 20 µmoles F 1 6 P was added and the system incubated at 37° for another 20 minutes The solution was next transferred to a cuvette and the recorded reaction started at zero time by the addition of 0 5 µmoles DPNH The addition of 2 µmoles $CuSO_4$ completely stopped this reaction as performed The addition of 5 µmoles $MgCl_2$ did not significantly change the reaction

Glucokinase was also demonstrated in cell-free extracts by the synthesis of G-6-P from glucose in the presence of ATP The G 6-P was identified by paper chromatography

A similar phosphorylation of fructose, ribose, ribulose or δ gluconolactone did not take place This seems to indicate a strict substrate specificity of the glucokinase activity

Phosphohexoisomerase The data presented in Fig 4 also demonstrate an activity corresponding to the enzyme phospho-fructo-glucoisomerase The existence of such a hexoisomerase was also indicated by paper chromatography From a reaction mixture which contained cell-free extract from N meningitidis and G-6-P, F 6 P was identified after 30 minutes incubation The reverse reaction could also be demonstrated by supplying F-6-P and noting the formation of G-6 P

Like the chemical assay, the photometric method by means of a coupling of the phosphohexoismerase with the G-6 P dehydrogenase demonstrate no activity corresponding to a fructokinase

Aldolase Cell-free preparations from meningococci showed a formation of triosephosphate from F-1, 6-P in a system where the oxidation was intercepted by hydrazine as trapping agent in the way it has been

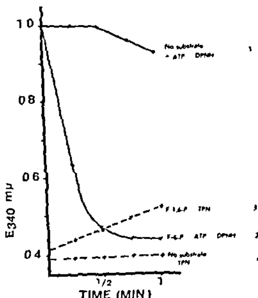


Fig 6

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Experiments were also performed in which the system with α glycerophosphate dehydrogenase and triosephosphate isomerase was used for the analysis of triosephosphate from reaction mixtures where protein had been precipitated in the way it has been described under methods

The DPNH oxidase effect of crude extracts which is apparent from the data of Fig 5 could be completely inhibited by the addition of 1 to 2 μmoles KCN The carbonyl trapping activity of cyanide in these concentrations did not significantly influence the reaction as performed An orientation concerning the influence of metal ions on the aldolase activity indicate a pronounced inhibition by copper Iodacetate or cysteine does not inhibit Thus, the aldolase in N meningitidis approaches that of liver more than that of yeast in its properties The activating effect of inorganic phosphate which appears from the experiment re-

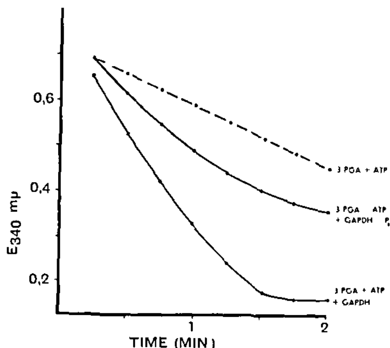


Fig 7

Activity of phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase in cell free extracts from N meningitidis Meningococcus extract dilution 0 5 ml was incubated at room temperature for 7 minutes with the following mixture 100 μmoles barbital buffer pH 8, 60 μmoles cysteine, 5 μmoles $MgCl_2$, 2 μmoles ATP, 5 μmoles glycine, 3 2 μmoles KCN, 0 2 mg GA-3 P dehydrogenase Then 0 5 μmoles DPNH was added and the reaction proper started by the addition of 20 μmoles 3 PGA as the potassium salt The phosphate experiments contained 100 μmoles P_i

corded in Fig 5 may be the result of an inhibition of the F-1,6-P phosphatase described below

Fructose-6-phosphate kinase and fructose-1,6-diphosphate phosphatase An activity corresponding to F-6-P kinase was also constantly present in extracts from meningococci in the way it has been demonstrated *in Fig 6*

Meningococci obviously also contain a F-1,6-P phosphatase The incubation of this substrate without ADP in the presence of cell-free extracts, releases inorganic phosphate and produces F-6-P The latter substance was identified in chromatograms where it occurred together with G-6-P, obviously produced from F-6-P through the effect of the hexoisomerase A reduction of TPN after the incubation of F-1, 6-P with meningococcus extract also is in agreement with the assumption of a phosphatase activity This effect appears from the data reported in Fig 6 The effect was also demonstrated in experiments where F-1,6-P was incubated with meningococcus extract and the reaction interrupted by protein precipitation with perchloric acid The supernatants from these precipitations after neutralization reduced TPN in the presence of cell-free extracts from the meningococci

When crude extracts were tested this phosphatase appeared more active in old cells preserved in the frozen state than in new ones. This eventually was found to be the result of a progressive destruction of the enzyme aldolase during storage. In extracts from new cells the aldolase favorably competed for the common substrate F-1, 6-P.

A better system for the measurement of the phosphatase activity could be obtained by the addition of copper which inhibits the aldolase in the way it has previously been described.

Phosphoglycerate kinase. Phosphoglycerate kinase activity was assayed for in a system where the oxidation of DPNH in the presence of GA-3-P dehydrogenase was used as the indicator system for the generation of 1,3 diphosphoglyceric acid. This has been shown in Fig. 7. The kinase activity was high in all extracts tested.

Glyceraldehyde-3 phosphate dehydrogenase. The bacterial extracts had an activity corresponding to a GA-3-P dehydrogenase. This was demonstrated in the system shown in Fig. 7, but could also easily be indicated by a reduction of DPN with GA-3-P in the presence of inorganic phosphate. The role of P_i in this reaction was also demonstrated by a marked phosphate inhibition of the reaction from 3-PGA through 1,3-PGA to GA-3-P. This effect has been illustrated in Fig. 7 where the addition of P_i results in a pronounced inhibition of the complete system.

When the GA-3-P dehydrogenase and PGA kinase reactions with meningococcal extracts were run from 3-PGA to GA-3-P the dehydrogenase reaction appeared as the rate limiting factor. The relative rates of DPNH oxidation in crude extracts, and in extracts to which excess of the GA-3-P dehydrogenase had been added appear from the data recorded in Fig. 7.

or

...ogen was incubated with 10 μmoles potassium salt of PEPA, 10 μmoles ADP and 2.5 μmoles $MgCl_2$ in 70 μmoles tris buffer pH 7.4, 2.77 μmoles pyruvate was generated after 10 minutes and 4.16 μmoles after 20 minutes at 37°.

Phosphoglycerate mutase and enolase. In a following series of analyses meningococcal extracts were analysed for an enzymatic transfer corresponding to the remaining part of the Embden-Meyerhof pathway. In agreement with the conventional route a synthesis of pyruvate occurred when 3-PGA was incubated with ADP in the presence of meningococcus extract. In the system used for the demonstration of the PEPA transphosphorylase the PEPA was replaced by 20 μmoles potassium salt of 3-PGA. After 15 minutes incubation at 37° 1.18 μmoles pyruvate had been synthetized, and after 20 minutes this amount was increased to 1.57 μmoles.

DISCUSSION

N. meningitidis which is adapted to growth with glucose as the sole source of carbon and energy appears capable of oxidizing glucose and several phosphorylated compounds which are usually found as intermediates in the pathways of glucose catabolism. The observed values for oxygen consumption, however, never reach the stoichiometric amounts required for the complete oxidation of the theoretical amount of substrate to CO_2 and H_2O. Analyses of the over-all reaction indicate that resting cells of *N. meningitidis* oxidize glucose to yield approximately 4 μmoles CO_2 and 1 μmole acetic acid per μmole substrate with RQ close to one when the oxidation is optimal judged by the oxygen uptake.

This clearly indicates that glucose is oxidized further down than to the acetate level. The present findings agree with the assumption that the "primary" oxidative pathway of glucose is rapid and complete. The "secondary" oxidative pathway, however, is not complete, since only approximately half the acetate which—hypothetically—should be generated by a "primary" pathway of glucose oxidation is completely oxidized to CO_2 and H_2O. The total oxidation of glucose by resting cells of *N. meningitidis which have been adapted to the use of glucose as only* source of energy and carbon may on the whole approach the following stoichiometry under optimal conditions:



$$C_6H_{12}O_6 + 4\ O_2 = CH_3COOH + 4\ CO_2 + 4\ H_2O$$

N. meningitidis apparently is equipped with the enzymes necessary in order to perform glycolysis according to the conventional Embden-Meyerhof route since activities could be demonstrated in cell-free extracts corresponding to the main substrate transfers of this pathway.

Activities of some key reactions of the Embden-Meyerhof route were also compared in extracts from "wild type" meningococci, and in extracts from meningococci adapted to growth on the minimal medium. The only activity in which a significant change occurred after the adaption was the glucokinase which increased nearly to the double after the adaption.

Other pathways of "primary" glucose oxidation will be discussed in a following publication.

SUMMARY

The over-all pattern of glucose oxidation by resting cells of *Neisseria meningitidis* adapted to growth on a minimal medium with glucose as the sole source of carbon and energy has been described.

Activities which correspond to the following enzymes related to the Embden-Meyerhof pathway of glycolysis were demonstrated *in cell-free* extracts:

Glucokinase	Triosephosphate isomerase
G 6 P dehydrogenase	GA 3 P dehydrogenase
Phosphohexoisomerase	PGA kinase
F 6 P kinase	PGA mutase and enolase
F 1 6 P phosphatase	PI PA transphosphorylase
Aldolase	

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DISCUSSION

N meningitidis which is adapted to growth with glucose as the sole source of carbon and energy appears capable of oxidizing glucose and several phosphorylated compounds which are usually found as intermediates in the pathways of glucose catabolism The observed values for oxygen consumption, however, never reach the stoichiometric amounts required for the complete oxidation of the theoretical amount of substrate to CO_2 and H_2O Analyses of the over-all reaction indicate that resting cells of *N* meningitidis oxidize glucose to yield approximately 4 μmoles CO_2 and 1 μmole acetic acid per μmole substrate with RQ close to one when the oxidation is optimal judged by the oxygen uptake

This clearly indicates that glucose is oxidized further down than to the acetate level The present findings agree with the assumption that the "primary" oxidative pathway of glucose is rapid and complete The "secondary" oxidative pathway, however, is not complete, since only approximately half the acetate which—hypothetically—should be generated by a "primary" pathway of glucose oxidation is completely oxidized to CO_2 and H_2O The total oxidation of glucose by resting cells of N meningitidis which have been adapted to the use of glucose as only source of energy and carbon may on the whole approach the following stoichiometry under optimal conditions



$$C_6H_{12}O_6 + 4\,O_2 = CH_3COOH + 4\,CO_2 + 4\,H_2O$$

N meningitidis apparently is equipped with the enzymes necessary in order to perform glycolysis according to the conventional Embden-Meyerhof route since activities could be demonstrated in cell-free extracts corresponding to the main substrate transfers of this pathway

Activities of some key reactions of the Embden-Meyerhof route were also compared in extracts from "wild type" meningococci, and in extracts from meningococci adapted to growth on the minimal medium The only activity in which a significant change occurred after the adaption was the glucokinase which increased nearly to the double after the adaption

Other pathways of "primary" glucose oxidation will be discussed in a following publication

SUMMARY

The over-all pattern of glucose oxidation by resting cells of Neisseria meningitidis adapted to growth on a minimal medium with glucose as the sole source of carbon and energy has been described

Activities *which correspond* to the following enzymes related to the Embden-Meyerhof pathway of glycolysis were demonstrated in cell free extracts

Fellowes & Routh (1944) and *Oeding* (1954) examined extraction methods which had been used on Gram-negative intestinal bacilli, and the hot formamide method. The latter was found to give the most satisfactory yield of polysaccharide material. *Oeding* (1957 b) examined his fractions by the indirect hemagglutination technique. He found evidence of a labile grouping on the polysaccharide responsible for the sensitizing ability.

A more extensive review of these and other earlier investigations, and a critical evaluation of the methods used, have recently been given by *Oeding* (1960).

Oeding's fractions were obviously not pure, and the results obtained by the hemagglutination technique claimed for more gentle methods. The aim of the present work has been to get a basis for further and more detailed investigations of staphylococcal antigens by immunochemical methods. The strain used by *Oeding* (1954) was known to possess a type-specific polysaccharide antigen. The choice of strain 1503 (*Oeding* 1952) for the present work has two reasons. First, the strain possesses a type-specific protein antigen, the *a* antigen, which can easily be detected by agar precipitation (*Haukenes & Oeding* 1960). Second, the strain has not been found to contain type specific carbohydrates (*Oeding* 1953). Various extraction and fractionation methods have been attempted, but the fractions have not been examined chemically unless the yield has been satisfactory and the antigens probably not denatured.

While *Julianelle & Wieghard* (1934) and *Verwey* (1940) used the term type specific for antigens common to either yellow or white staphylococci, we have found it more convenient to designate these antigens as group specific, and antigens which are present only in some coagulase positive strains, as type-specific.

METHODS

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83 by [illegible]
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The bacteria were grown on nutrient agar in Roux bottles and harvested in saline or a buffer. The yield of 100 Roux bottles varied from 80 to 100 g wet bacteria.

Immune sera were produced by intravenous injections of formal[illegible]

Molisch test. To 5 drops of the sample were added 2 drops of 1 per cent alpha naphthol (Merck) in ethyl alcohol. Three ml of concentrated H_2SO_4 [illegible]
carefully [illegible]
mixing th[illegible]
is theref[illegible]

Biuret [illegible]
over whi[illegible] per cent $CuSO_4$

FROM UNIVERSITY OF BERGEN, SCHOOL OF MEDICINE THE GADE INSTITUTE
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EXTRACTION AND FRACTIONATION OF ANTIGENIC MATERIAL FROM STAPHYLOCOCCUS AUREUS

By

GUNNAR HAUKENES, NORVALD LOSSIGARD and PER OEDING

Received 16 1 61

Compared with the extensive chemical and immunological investigations which have been carried out on the antigens of pneumococci and streptococci, little is known of the nature of staphylococcal antigens

Julianelle & Wieghard (1934, 1935 a and b) isolated two staphylococcal polysaccharides, type A and type B, from virulent and avirulent strains respectively The two substances differed in chemical composition, were group-specific by precipitation, and non-antigenic in rabbits The bacteria from 24 hour old broth cultures were extracted with N/16 hydrochloric acid at 100° C for 20 minutes Insoluble material formed by dropwise addition of 40 per cent sodium hydroxide to neutrality was removed The aqueous extract of the precipitate formed with ethyl alcohol was precipitated with trichloroacetic acid to remove remaining protein The resulting type A substance contained 4 1 per cent nitrogen, 6 27 per cent phosphorus, and about 25 per cent reducing sugars after hydrolysis The Molisch reaction was strongly positive (*Wieghard & Julianelle* 1935) More detailed examinations of this polysaccharide have not been reported

Verwey (1940) examined *Julianelle & Wieghard*'s type A strain by a milder technique Disintegrated cells were extracted with water, and the nucleoproteins were precipitated with hydrochloric acid Trichloroacetic acid was added to the supernatant, and the substance thus obtained was found to be a protein containing only traces of phosphorus and carbohydrate The polysaccharide fraction was then precipitated with alcohol The nucleoproteins cross-reacted with sera against avirulent staphylococci, while the protein and polysaccharide reacted only with the homologous serum The distribution of the protein antigen among virulent staphylococci was not examined

Staphylococcal nucleoproteins have been found to cross-react with other Gram-positive cocci (*Lancefield* 1925 *Boor & Miller* 1934)

Another cross-reacting substance has been isolated by *McCarty* (1959) and identified as a glycerophosphate compound

terials Serologically the pH 5 0 precipitate was negative and the pH 4 4 precipitate weakly positive, while the alcohol precipitate was strongly positive by the ring test (Table 1)

The amount of acid-precipitated material is very small, while the alcohol-precipitated material is more easily extracted Freezing and thawing is thus not an efficient method for the disintegration of staphylococci Further purifications and chemical analyses of the fractions were not performed from this batch

Urea Extraction

Ten grams of dried bacteria was moistened with water and mixed with twice its weight of urea The mixture was incubated at 37° C for 70 hours and then extracted with water (*Seibert & Fabrizio* 1952) Acid- and alcohol precipitated materials showed approximately the same composition as in the previous experiment, and the method possessed no advantage over freezing and thawing Moreover, the process undoubtedly involves risk of degrading protein antigens

TABLE 2
Composition of Fractions after Grinding in Ball Mill

Fractions	Nitrogen per cent	Phosphorus per cent	Reduc sugars per cent	Biuret 1 mg ml	Molisch 1 mg ml	Dische 1mg ml	Bial 1 mg ml	Ring test 1 mg ml
Precipitate at pH 4.2	11.36	2 30	6.35	++	+ (+)	+++	++	1 17
Alcohol precipitate	5 50	4 82	37 2	+	—	—	—	1 5 000

Grinding in Ball-Mill

Washed and acetone dried bacteria were grown in a ball mill with steel balls at room temperature Smears were taken at intervals After three hours' grinding only scattered intact cells could be seen Towards the [illegible]
an[illegible]
ext[illegible] of organisms achieved a dark brown colour smelt like herring pickle, and the steel balls were dark spotted

Several portions of bacteria were examined, and the extracts were precipitated with *acid at pH 4 2* and then with ethyl *alcohol*

The chemical data of the acid precipitate (*Table 2*) correspond well to the nucleoprotein fraction isolated by *Sevag et al* (1938) from streptococci The alcohol *precipitate* shows resemblance with *Julianelle & Wieghard*'s carbohydrate A, but a striking disagreement is [illegible] gative Mol[illegible]

[illegible] those
[illegible] per cent of

Bial's orcinol test (1902) was used for the demonstration of pentoses and *Dische's* diphenylamine test (1930) for desoxyribose

Estimation of phosphorus was carried out according to *Fiske & Subbarow* (1925) with some of the modifications of *Youngburg & Youngburg* (1930)

Reducing sugars after hydrolysis with 3 N HCl at 100° C for 3 hours were estimated by the Hagedorn Jensen method (*Kabat & Mayer* 1948)

Nitrogen determination was carried out by the micro Kjeldahl method as described by *Kabat & Mayer* (1948), with the exception that N/70 HCl was prepared by titration against Na CO_3

Hexosamine was determined by the method of *Rondle & Morgan* (1955) after acid hydrolysis (see reducing sugars)

Dialysis was performed in cellophane tubes against running tap water or against distilled water in the refrigerator

Dry weight was determined after drying in vacuo over silica gel

All extraction and fractionation procedures have been carried out in the cold

RESULTS

Freezing and Thawing

Ten grams of washed and acetone-dried bacteria of strain 1503 was suspended in 40 ml of distilled water and frozen in a mixture of carbon dioxide ice and acetone followed by rapid thawing This process was repeated 28 times, but a Gram-stained smear showed no microscopic evidence of disintegration The organisms were then centrifuged and extracted thrice with 20 ml portions of water in the refrigerator The extracts, total 100 ml, were combined The Molisch and biuret reactions were positive in *dilution 1 10* The ring test was weakly positive in 1 1,000 dilution of the extract

Further extraction was attempted by subjecting the frozen and thawed organisms to saturated sodium chloride solution and water alternately After two extractions only small amounts of serologically active material were demonstrated by the ring test A smear showed still no signs of disintegration of the cells

TABLE 1

Composition of Fractions after Freezing and Thawing of 10 g of Dried Staphylococci

Fractions	Yield *mg*	Nitrogen *per cent*	Phosphorus *per cent*	Reduc sugars per cent	Ring test
Precipitate at pH 5 0	85 1	13 0	1 45	13 1	+
Precipitate at pH 4 4	47 7	10 8	1 74	10 6	+
Alcohol precipitate	127 5	6 3	5 77	19 9	+++

The extracts were precipitated with hydrochloric acid at pH 5 0 and pH 4 4 successively The supernatants were precipitated with four volumes of ethyl alcohol, and the aqueous extract of the precipitate was again acidified to pH 4 4 to remove rests of acid insoluble material Similar fractions from each extract were combined and dialyzed before dry weight estimation and chemical analyses The biuret test was positive in all three fractions, strongest in the acid precipitated ma-

layered gauze before centrifuging A control experiment was made using the same procedure, but without bacteria The centrifugates had the same dark yellow colour in both instances and were strongly positive to the Molisch and biuret tests It is therefore evident that the first washing contains great amounts of material extracted from the culture medium

The effect of washing and defatting was studied more detailed The growth from 10 Roux bottles was harvested in 125 ml of saline and washed twice in 60 ml portions of saline, thus obtaining "extracts" 1, 2 and 3 The bacteria were then extracted twice for two hours with acetone, and again washed thrice in saline, giving "extracts" 4, 5 and 6 Thereafter the organisms were extracted overnight with N/100 sodium hydroxide and the next day once more with alkali for three hours The eight "extracts" thus obtained were examined by the Molisch and biuret reactions and by ring test precipitation

TABLE 3
Composition of Washings

Extracts number	Colour	Ring test	Biuret test	Molish test
1	Dark yellow	+ 1 100	++	+++
2	Yellow	+ 1 100	(+)	(+)
3	Colourless	+++ 1 10	—	(+)
4	Yellow	+++ 1 10	+	+
5	Colourless	+++ 1 10	(+)	(+)
6	Colourless	+++ 1 10	—	—
7	Yellow	+++ 1 10	+(+)	+(+)
8	Colourless	+++ 1 10	(+)	(+)

It appears from Table 3 that measured by the ring test titers most of the serologically active material is extracted during the first two washings Further investigations of the extracted antigens ha e been

... serological typing of staphylococci (*Haukenes*, unpublished data) Some of the results will be given here A thick suspension of crushed strain 1503 bacteria and undiluted antiserum 1503 were placed in each basin Three characteristic precipitation lines developed easily in addition to some faint and more inconstant lines Near to the serum basin a line appeared after one to two days ...

... Near the antigen basin the sharp *n* line described by *Haukenes & Oeding* (1960), is found The strong and

hexosamine calculated as glucosamine, while the hexosamine content of the alcohol precipitate varied from 16 to 20 per cent

We have thus got the same fractions as in the foregoing experiment, the acid precipitate and the acid soluble, alcohol precipitate The fractions differ considerably serologically and in chemical composition In the grinding experiments, however, the amount of acid precipitate was much larger than after freezing and thawing, about 1 5 g from 10 g of dried bacteria This is obviously a result of disintegration of the cells with release of nucleoproteins The corresponding amounts of alcohol precipitate varied from 25 to 50 mg

Grinding in a ball mill gave a satisfactory yield However, the brown colour of the mass, the peculiar smell, and the discoloration of the steel balls suggest some kind of degradation, possibly a reaction between the steel and sulphur-containing constituents of the cells The brown colour of the extract followed the acid precipitate, and a brown pigment could be separated by heating to 56° C for 30 minutes in alkaline solution The pigment dissolved readily in hydrochloric acid below pH 1 0, and showed no fluorescence Spectrophotometric examination revealed a peak at 310 mμ The pigment was also readily dissolved in acetic acid and changed to a more reddish colour

Various fractionation methods were attempted, *e g* salting out with ammonium sulphate and sodium chloride, precipitation with ethyl alcohol and acetone at different concentrations, shaking with chloroform (*Sevag* 1934), and phenol extraction It was not by these methods possible to achieve distinctly separated fractions The results were difficult to reproduce, *e g* by the first addition of hydrochloric acid the bulk of the acid-precipitable material came down at pH 4 2, whereas this fraction by re-precipitation could be separated in several fractions at various pH values The same thing was observed after heating to 56° C at alkaline reaction to remove the pigment This can probably be explained as a rapid degradation of the nucleoproteins, especially when pH is raised to the neutral point to dissolve the precipitates Heating to 56° C in alkali undoubtedly results in a splitting of the nucleoproteins in nucleic acids and proteins (*Sevag et al* 1938) The alcohol precipitate, on the other hand, seemed very stable

Thus our grinding technique and subsequent fractionations were found to involve degradation Grinding at lower temperature with stone balls instead of steel balls is to be preferred

Precipitation of the fractions in agar will be described in connection with the next experiment

Washing of the Bacteria

As a routine the bacteria from a solid medium are suspended in saline and spread over the nutrient agar surface in Roux bottles Superfluous fluid is removed After 18 hours incubation, the growth of each Roux bottle is harvested in 10 ml of saline and filtered through four-

found that about 80 per cent of the cells was disintegrated by this procedure.

In one of our experiments 67 g of wet bacteria was crushed and then extracted with 600 ml of isotonic buffer at pH 7.0 overnight. The extract was yellow and opalescent. The amount of material extracted was 2,060 mg. The crude extract was precipitated at pH 4.2 by dropwise addition of N hydrochloric acid under constant stirring, constituting the acid precipitate. After reprecipitation the fraction was dialyzed. The supernatant after acid precipitation was adjusted to pH 5.2, and four volumes of ethyl alcohol were added. The alcohol precipitate was dissolved in water and precipitated with hydrochloric acid at pH 4.2. This process was repeated until no precipitation followed the addition of hydrochloric acid. The acid precipitates thus obtained were combined and designated acid precipitate from the alcohol fraction. The alcohol supernatant was distilled under reduced pressure to remove the alcohol.

Chemical analyses of acid- and alcohol-precipitates from different batches showed the same composition of the fractions as in the foregoing experiments. The amount of the alcohol fraction varied, but it was regularly much greater than in the experiment cited in Table 5. The alcohol precipitate was found to contain about 30 per cent of reducing sugars after hydrolysis, from 4 to 6 per cent of nitrogen, and 5 to 7 per cent of organic phosphorus. The Molisch and biuret reactions were weakly positive. The chemical composition of the acid precipitate was suggestive of nucleoproteins. The *n* antigen (*Haukenes & Oeding* 1960) is found in the acid precipitate.

TABLE 5

Antigenic Patterns of Crushed Bacteria and Fractions from Extract of Crushed Bacteria

Material	Yield mg	Ring test 1 mg/ml	Agar precipitation principal lines
Crushed bacteria			Polysaccharide line Line corresponding to *Jensen*'s antigen A *n* line
Acid precipitate	1765	(+) 1:10	No polysaccharide line Line corresponding to *Jensen*'s antigen A *n* line
Alcohol precipitate	23.4	(+) 1:5000	Strong polysaccharide line Faint line corresponding to *Jensen*'s antigen A No *n* line
Acid precipitate from alcohol fraction	58.8	++ 1:10	No polysaccharide line Strong line corresponding to *Jensen*'s antigen A Faint *n* line
Alcohol supernatant	16.8	+ 1:100	Polysaccharide line only

often splitted band between these two lines corresponds to *Jensen*'s (1958) antigen A.

The growth of 101 Roux bottles was harvested in 880 ml of isotonic phosphate buffer of pH 7.0, centrifuged, and washed in the same volume of buffer. The two washings were designated extract 1 and 2 respectively. Both extracts were fractionated as before giving the acid precipitate, alcohol precipitate, and alcohol supernatant. The fractions were dialyzed before the dry weight was estimated and the ring test and the Molisch and biuret reactions performed.

The two extracts were examined by agar precipitation. In extract 1 the line representing the polysaccharide was strong and could be demonstrated after diluting the extract to 1:10. In extract 2 this line was faint. The band representing *Jensen*'s (1958) antigen A, was more pronounced in the first than in the second extract, and the *n* line was only found in the first extract.

It is evident from Table 4 and the agar precipitation experiments that most of the serologically active material is found in the first extract. This extract also contains great amounts of material from the medium. The time allowed for extraction was, however, much longer for the first extract than for the second one, since some time elapsed before all the bacteria from the Roux bottles were collected and centrifuged. Time seems to be an important factor in extraction (cf. Table 3, where the extraction time was the same for the two first extracts). To reduce the loss of antigenic material during washing it is therefore important that the bacteria are centrifuged immediately after being suspended in the fluid.

The polysaccharide is easily extracted from intact bacteria indicating a superficial location. It is important to note, that we have not been able to demonstrate antigens in the washings other than those found in washed and crushed bacteria.

TABLE 4

Fractions Obtained from two Successive Washings

Fractions	Yield mg	Ring test titers 1 mg/ml	Biuret 1 mg/ml	Molisch 1 mg/ml
Extract 1	3 488			
Extract 2	217			
Acid precip. from extr. 1	1 299	1:120	(+)	++
Acid precip. from extr. 2	32.2	1:150	(+)	+
Alcohol precip. from extr. 1	1 110	1:20		+++
Alcohol precip. from extr. 2	49.6	1:100		+++
Alcohol sprnt. from extr. 1	73.2	1:10	(+)	+
Alcohol sprnt. from extr. 2	27.3	1:10		+

Crushing in a Bacteria Press

Wet bacteria were frozen and crushed in a Shandon Hughes bacteria press, which had been cooled down to —30° C before use. *Hughes* (1951)

dation The use of such methods as multiple enzymes electrophoresis and ion exchange chromatography may render isolation of pure and native antigens from *Staph aureus* possible

SUMMARY

Different methods for disrupting the bacteria of a certain strain of *Staph aureus* have been examined Crushing in a bacteria press was found to be the method of choice

Whole and disrupted bacteria have been extracted and the extracts examined serologically before and after fractionation with hydrochloric acid and alcohol Some chemical data of the fractions are given The chemical composition of a polysaccharide antigen has been discussed and it is presumed to be an amino sugar compound It is obvious that the nucleoproteins soon undergo degradation and fractionation procedures should be performed with great care The agar precipitation method was found to be useful as a lead during extraction and fractionation procedures

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Of the methods examined, disruption in the bacteria press is best suited, giving a satisfactory yield without obvious signs of degradation

DISCUSSION

The antigenic structure of pyogenic staphylococci is obviously very complex (*Oeding* 1952 and 1953, *Stern & Elek* 1957) *Oeding* (1953) has shown that some agglutinogens are heat-stable and resistant to trypsin, while others are heat-labile and destroyed by trypsin Extracts of the bacteria should be expected to contain these type-specific antigens of carbohydrate or protein nature

The method used by *Wieghard & Julianelle* (1935) to isolate and purify carbohydrate A, includes precipitation of the active material by alcohol and removal of proteins by trichloroacetic acid It is not probable that this procedure will separate different carbohydrate antigens The composition of the resulting product will therefore depend on the strains chosen The same objections can be made to other fractionation methods such as precipitating nucleoproteins by hydrochloric acid and other proteins by trichloroacetic acid

In our experiments the alcohol precipitate shows great resemblance to *Julianelle & Wieghard's* carbohydrate A The discrepancy as to the Molisch reaction is not easy to explain Agar precipitation experiments with our fraction disclosed at least two antigen-antibody systems Other carbohydrate antigens, giving a positive Molisch reaction, may complicate the investigations The Molisch reaction was controlled quantitatively (*Kabat & Mayer* 1948) using glucose as a standard, and the carbohydrate content of the alcohol fraction was found to be 2 to 3 per cent The Molisch reaction is negative in 2-amino sugars, while other amino sugars are found to behave as simple monosaccharides (*Kent & Whitehouse* 1955) The hexosamine content of the polysaccharide was 16 to 20 per cent calculated as glucosamine It is therefore probable that the polysaccharide is a 2-amino sugar compound Further purification by other methods is needed before the antigen can be analyzed more detailed

The great amount of material precipitated with acid, contains several antigenic components Nucleoproteins are likely to constitute the major part of the fraction, but in addition a type-specific protein was found

Serological typing of staphylococci is based upon agglutination reactions (*Oeding* 1952), and characteristic precipitation lines in agar have been found representing some of these agglutinogens (*Haukenes & Oeding* 1960 *Haukenes*, unpublished data) The serological basis for immunochemical studies of staphylococci is, however not satisfactory and it is therefore important to choose strains with known and easily detectable antigens

It is evident from these experiments that chemical extraction and fractionation methods should be used with great care to avoid degra-

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ANTIBODY AGAINST NORMAL EGG MATERIAL RESULTING FROM INFLUENZA VACCINATION

By

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Received 12 ii 61

The influenza virus contained in vaccines is produced in the cells lining the allantoic cavity of chick embryos, and it is probably inevitable that the vaccine preparations contain some normal egg material, free or attached to the virus particles One would therefore expect that antibody against normal egg material would also be produced in response to an injection of influenza vaccine This antibody, however, is not demonstrable by conventional methods In the present investigation a technique was used which revealed the formation of antibody capable of reacting with normal egg allantoic fluid in several of the vaccinated individuals

MATERIALS AND METHODS

Influenza vaccines Two preparations were used both monovalent A_2 vaccines without adjuvants One was a commercial preparation received from U S A the other was made by the authors Both vaccines had been produced according to standard procedures using the virus infected allantoic fluid from hens eggs The commercial preparation A_2 Japan/305/57 of high antibody sensitivity had been purified by absorption to chick red cells and subsequent elution It contained 400 CCA units per ml Micro Kjeldahl gave 0 2 mg N per ml The authors preparation A_2 Singapore 1/57 of medium antibody sensitivity had been purified by differential centrifugation the haemagglutinin content was half of that of the commercial micro Kjeldahl showed 0 03 mg N per ml Formal [illegible]

[illegible] uary 1960 40 [illegible] the commercial preparation 40 the authors The rest of the company 57 individuals who where distributed equally in the same rooms as the vaccinees served as controls they got no placebo The vaccination was voluntary

The authors are indebted to Major A Borgersen Major H Hammer and Captain H Frang of the Army Medical Corps Training Cen[illegible]

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In 63 of the vaccinees A_2-antibody was found already in the sample drawn before vaccination. In these the titres of the subsequent samples were usually high, while they were low in 14 with no detectable amounts of basic A_2-antibodies. In 3 individuals without detectable basic A_2-antibodies the antibody rise was similar to that of the former 63, so that these 3 probably also had previous contact with this virus. Thus there was serological evidence of previous A_2-infection in 66 of 80 recruits, i.e. 83 per cent, as none of them had been vaccinated against influenza previously.

When for the 80 vaccinees the A_2-titres in the prebled and in the 10 days sample were compared in a scatter diagram, the picture of two different populations emerged: previously infected and previously uninfected.

Examinations of heterologous antibody development. The sera were examined in HI tests with various A, A_1 and B strains in order to learn whether the vaccination had resulted in heterologous antibody development. In the following the percentages showing antibodies are noted in the brackets. With swine influenza virus (5), WS (24) and PR 8 (48) no individuals showed a significant increase. With FM 1 (94) there was one individual (recruit No. 15) and with A_1/Netherlands 3655 (56) two (Nos. 15 and 106) who presented a significant increase. The latter strain was received from *J. Mulder & N. Masurel*.

An anamnestic response might offer a plausible explanation of the increase of these antibodies. However, even with B strains as test antigens a significant increase was observed. With Lee (70) as many as 7 of the vaccinated individuals presented a significant increase in one particular titration, and in 4 of them this was reproduced in two additional independent titrations. 3 vaccinated individuals presented a significant increase to B Bon (83), 2 of these were among the 4 with an increase to Lee. As B strains in any case are much less related antigenically to A_2-strains than A and A_1 strains are, the more frequent heterologous increases to the former were surprising.

These increases could hardly be ascribed to random [illegible]

[illegible] distinct HI titre increase to a B-strain were examined in a CF test with soluble B antigen without any of them showing as much as a twofold increase. The absence of a significant HI titre increase to the B strains in all but one (No. 153, discussed later) of the unvaccinated controls speaks also against prevalence of B infections in the recruit company during the trial. — Increase to B strains was seen with both vaccine preparations; a contamination of both with B virus seemed improbable.

The following experiments, however, gave the explanation of the peculiar B-antibody increase which was more or less regularly observed in some of the A_2-vaccinated individuals in the HI tests described above.

and about half a dozen refused Allergic individuals were not vaccinated Those who did not complete the trial have not been included in the numbers above

Serum samples were drawn from vaccinees and controls 4 days before and about 20 days after the vaccination from the vaccinees also 10 days after vaccination

Haemagglutination inhibition (HI) test The virus used for testing A_2 antibodies was an egg-ferret-mouse egg line of A_2/Japan/305/57; this virus has a high antibody sensitivity (5) Tests were also performed with A-, A_1- and B strains Except when noted the antigens consisted of diluted allantoic fluid from infected eggs In one experiment the antigen used was tracheo-bronchial washings from mice infected 4 days previously by intranasal instillation under ether anaesthesia of 0 1 ml of Lee-infected, egg allantoic fluid diluted 10^{-2}.

All sera were pretreated with cholera filtrate in order to destroy normal inhibitors (9) The antihaemagglutinins then observed were considered to be antibodies although the complete removal of interfering, normal inhibitor activity was not strictly proven All sera were absorbed with fowl red cells in order to remove haemagglutinins The HI-test was performed by the World Influenza Centre's plastic plate technique (4) The fowl red cells used were particularly sensitive to antibodies inhibiting B-strains Serum titres are given as primary dilutions after addition of the cholera filtrate, and correspond to 4 haemagglutinating doses of virus

A modification of the HI-test, a haemagglutination inhibition neutralization (HIN) test, which made it possible to demonstrate antibody reacting with normal egg material will be described later in this paper

Complement fixation (CF) test Soluble antigens were prepared from chorioallantoic membranes of eggs infected with PR 8 or B/Denmark/1/59, a strain received from *P von Magnus* and *K Birlum Petersen* For the preparation WHO Method A (10) was used, modified by spinning finally for 1 hour at 25 000 G and absorbing with red cells to ascertain a practically complete removal of viral haemagglutinin Optimal antigen dilutions were determined in two dimensional (chessboard) titrations against serial dilutions of PR 8 or Lee convalescent guinea pig serum, or anti normal membrane rabbit serum Also the CF test was performed in the plastic plates About 4 doses of complement were used, titres refer to 50 per cent haemolysis which was determined visually Fixation took place over night at +4° C

A fourfold titre difference was regarded as significant in the HI- and CF tests An individual was recorded as one developing a significant increase when a fourfold titre increase was observed between the first and the second or third serum sample When no inhibition, alt fixation was observed in the first dilution step (1 5), titre ratios were calculated on the assumption that the 50 per cent inhibition (fixation) would have occurred in the foregoing dilution (1 2 5)

RESULTS

Antibodies to A_2-haemagglutinin and to soluble A-antigen During the trial infections with A_2-virus were observed among the recruits 13 of the 57 unvaccinated controls developed a significant increase of the titres in the HI-test against A_2-virus, and or in the CF-test with soluble A-antigen Of the 80 vaccinated, 9 showed a significant increase in the CF-test against A, in vaccinated individuals this increase meant that infections might have taken place, though the possibility existed that the increase had been a response to the vaccine (1, 7) As a check the sera from individuals showing an increase with soluble A-antigen were also examined in a CF-test with normal, chorioallantoic membrane antigen with which they gave no fixation — The infected had mild or no clinical symptoms

The HI test with A_2 virus showed that 67 of the 80 vaccinated had developed a significant antibody increase, and that there was no substantial difference between those who had been vaccinated with the commercial preparation and those vaccinated with the authors'

participated in the trial and developed exceptionally high titres of antibody measurable with purified viruses only. He had been vaccinated with the commercial preparation while the five recruits had received the authors'. As with purified Lee the significant increases observed with purified swine influenza virus were abolished by normal allantoic fluid.

TABLE

Host Specific and Virus Specific Antibody Titres of Serum Samples Drawn before/10/20 Days after an Injection of Influenza A0 Vaccine. Host Specific Antibody Development Is Revealed by the HI Titre Increases Recorded with the Heterologous Purified Test Viruses

Person examined	Haemagglutination inhibition test with				Complement fixation test with soluble A antigen
	Lee red cell eluate	Swine influenza virus red cell eluate	PR 8 red cell eluate	A0 Japan 305 not purified	
No 1	<15 60/30	<5/10/<10	<10/<10/<10	<8/<8/<8	<5/<5 <5
No 55	<15/30/15	<10 20/20	20 20/20	<8/8 60	<5/15/25
No 104	<15 30/15	<10/<10/<10	<10/<10/<10	120 480/480	5 8 8
No 138	<15 60 45	<5/30/20	15/20/10	<8/10 20	<5/5/10
No 146	<15/45/30	<10/<10/<10	15/15 15	<8/<8/15	<5/<5 <5
[illegible]	30 360 60	<10 30/<10	20/40/—	<8 30 60	<5/<5/<5

In the following the antibody neutralized by normal allantoic fluid will be referred to as host specific antibody in contrast to virus specific antibody.

The host specific antibody had already reached its highest titres in the first (10 days) samples drawn after vaccination. The peak titre of the homologous virus specific antibody (anti A0) was usually reached in the first sample when basic antibody was present (secondary response) and in the second (20 days) sample when absent (primary response). As shown in the table host specific antibody titres can exceed virus specific (A0) titres in absolute values or relative increases. Swine influenza eluate gave lower titres than Lee eluate and PR 8 failed to reveal the formation of host specific antibody. Admittedly 4 of the 6 individuals tested with PR 8 eluate had some basic virus specific antibody against this strain and this may have masked the development of low titred host specific antibody which might otherwise have been detected with PR 8 eluate in these individuals.

About 20 vaccinated individuals possessed virus specific Lee antibody at such high titres that it might explain the absence of detectable amounts of host specific antibody inhibiting this strain. Of this group 15 individuals without basic antibody to swine influenza virus were tested with an eluate of the latter, and this disclosed in 3 individuals the formation of significant titres of host antibody neutralized by normal allantoic fluid. The 3 individuals had been vaccinated with

Antibody reacting with normal host material Knight (6) demonstrated that in rabbits, repeated injections of macromolecular material from normal egg allantoic fluid produced antibody which inhibited haemagglutination by influenza virus grown in chick embryos

In the present investigation the part played by antibody reacting with normal egg material in the observed titre increase to Lee was revealed in the HIN-test In this test the viral antigen had been purified by absorption to and subsequent elution from fowl red cells, washing the agglutinated cells with cold saline before elution 0 25 ml of serial, twofold dilutions of the sera from the vaccinated individuals were plated out in two parallel sets To one of them was added normal allantoic fluid, to the other saline, 0 25 ml in each hole The allantoic fluid had been harvested from 12 days old eggs and pretreated with equal amounts of cholera filtrate over night at 37° C followed by heating for 1 hour at 56° C and absorption with 10 per cent fowl red cells at 0° C After the allantoic fluid had been added to the serum dilutions, the plates were left on the bench for 1 hour (though a few minutes might have been sufficient) to allow the normal egg antigen to react with the antibody, subsequently 0 25 ml of 0 5 per cent fowl red cells and immediately afterwards 0 25 ml of 4–8 haemagglutinating doses of the purified virus were added and the patterns read as soon as the cells had settled

The result of testing with purified Lee virus sera from the vaccinated recruits was that in the absence of normal allantoic fluid (the saline parallel set) a significant antibody increase was observed in 7 individuals given the commercial vaccine, and in 18 given the authors' In the presence of normal allantoic fluid no significant increase was seen

In another test sera from the vaccinees who had shown an HI-increase with diluted, Lee-infected allantoic fluid were examined with such antigen in the presence of normal allantoic fluid, and this time no significant increase was observed

In agreement with these results was the finding that when an HI test was performed with mouse-grown Lee virus as antigen, none of the vaccinated recruits showed even a two-fold antibody increase In another test sera from five vaccinated individuals who had given an eight-fold or greater increase with egg grown Lee eluate (in the absence of normal allantoic fluid) were tested with mouse grown Lee eluate without showing any increase

6 of the 25 individuals with a significant increase recorded in the saline set with Lee eluate were among the 9 who showed a significant increase in the CF-test with soluble A-antigen

In another experiment PR 8 and swine influenza virus purified by red cell absorption-elution were used to test sera from five vaccinated recruits who had revealed a pronounced titre increase with purified Lee virus The results are recorded in the table which also presents the titre values given by a senior member of the company (Lt 1) who

as the unheated virus tested in parallel, although the heated virus was found to be 20 times as sensitive to normal inhibitor in human sera as the unheated

The possibility was considered that this individual might have received influenza vaccine even though he was recorded as unvaccinated, and that he had then responded with host specific antibody development However, with egg-grown Lee-eluate the titres were 5 20 in the presence as well as in the absence of normal allantoic fluid, and 5 15 with mouse grown Lee, so this explanation could be rejected

As he presented a CF-titre rise from < 5 to 20 with soluble A-antigen and an HI titre rise from < 8 to 120 with A_2-virus (he had no symptoms) and as other explanations seemed less valid, it was suggested that his Lee-titre increase was an anamnestic response to the A_2-infection (2)

DISCUSSION

How frequently a development of antibody against host material is recorded as a result of influenza vaccination, depends on three factors The antigenicity of the host component in the vaccine, the capacity of the individuals concerned to form these antibodies, and the sensitivity of the antibody test employed

As regards the sensitivity, no fixation was observed in CF-tests with normal chorioallantoic membranes or membranes infected with a heterologous influenza virus type (B) Further, when the HI tests were performed as usual with diluted, infected allantoic fluid constituting the viral antigen, the development of antibody attributable to normal host material was only occasionally observed When, however, purified virus was used instead, the development was seen quite frequently

By adding normal host material, in this case normal allantoic fluid, to the serum in the HI test the identity of the host specific antibody as such could be proven This modification of the HI test, the HI-neutralization test, had been developed in connexion with other studies, which will be described in a forthcoming publication (3) dealing [illegible]

[illegible] in the individuals concerned or resulting from the vaccination by a primary or anamnestic response

Another crucial point was the sensitivity of the test strain to the host specific antibody In the present investigation eluates of Lee, PR 8 and swine influenza virus were compared in tests on A_2-vaccinated individuals Lee was found to give the highest titres of host specific antibody but several individuals showed virus specific, basic antibody against this strain The host specific titres recorded with swine influenza virus were lower than with Lee, but very few had basic antibody against

the authors' preparation Their sera gave no complement fixation with soluble A-antigen

Because of the low sensitivity of the swine influenza strain to normal inhibitor in human sera it was possible to perform an HI-test with sera which had been pretreated only by heating for ½ hour at 56° C followed by red cell absorption The titres were compared with those obtained in a parallel titration with sera treated with cholera filtrate, heated and absorbed by the routine method It was then found that the type of pretreatment did not influence to any extent the ratio between titres before and after vaccination

Heterologous development of antibodies to A_1*-strains* It was mentioned above that 2 vaccinated individuals showed significant titre rise to A_1-virus in HI-tests with diluted, infected allantoic fluid No 15 gave the following titres against FM 1. 20/80 80 (prebled/10 days/20 days sample), against A_1/Netherlands/36 56 < 5/15/15 No 106 gave no rise against the former, against the latter < 5/10/10 No 15, who had received the commercial vaccine, did not reveal host specific antibody development when tested with purified Lee virus No 106 had received the authors' vaccine, and the titres with purified Lee were < 5 20 20, the increase was abolished by normal allantoic fluid Both showed a significant increase with soluble A-antigen No 15 < 5/10 —, No 106 < 5/30/15 They had no typical symptoms Anti-A_2 titres in the HI-test were No 15 < 5/< 5/10, No 106 < 5/< 5/80 Further studies with sera from No 15 could not be performed because the supply was exhausted, it was established only that the titre of the 10 days sample against FM 1 eluate was the same in the presence as in the absence of normal allantoic fluid As to No 106, A_1/Netherlands/36 56 eluate gave the same values in the presence as in the absence of normal allantoic fluid These findings suggest that the anti-A_1 titre increases observed in Nos 15 and 106 had not been caused by host specific antibody They might have been caused by A_2-infection, and their rapid development points to an anamnestic response

Heterologous development of antibody to a B-strain As noted above, one of the unvaccinated individuals, No 153, presented a significant titre increase when tested with diluted, Lee-infected allantoic fluid

Several explanations for this increase were examined First a test was made to see whether he had been infected with influenza B during the trial It was then found that he gave no complement fixation with soluble B-antigen, and no increase in HI-tests with three recent B-strains B Denmark/1/59 (< 8/< 8 in prebled/20 days samples), B Ann Arbor/1/59 (10 10) and B Utah/2/59 (10 10), the latter two strains obtained through the International Influenza Center for the Americas Thus a concurrent B-infection could not be demonstrated

Development of normal inhibitor which resisted treatment with the cholera filtrate could also be excluded An HI-test with Lee-infected allantoic fluid heated for ½ hour at 56° C gave the same titres (5 30)

been produced against virus host material In the same way purified swine influenza virus was inhibited by the host specific antibody

In addition to these findings various observations on host specific and virus specific antibody responses are described

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the swine strain PR 8 failed to reveal host specific antibody under the experimental conditions

None of the individuals showed host specific antibody before the influenza vaccination 21 of 40 individuals who received a single injection of an A_2-vaccine preparation made by the authors, developed detectable host specific antibody, as did 7 of 40 individuals who received a commercial one It was surprising that the former preparation obviously provoked more host specific antibody than the latter, which contained 7 times as much total nitrogen, and 2 times as much haemagglutinin as the former

This investigation does not reveal whether the production of antibody neutralized by normal host material was provoked by such material, or by viral material sharing antigenic determinants with its normal host (8), eventually by both In order to solve this problem one would perhaps have to inject normal host material under corresponding experimental conditions and amounts as the virus, and this experiment was not performed

It would also be interesting to know if allergic reaction to influenza vaccine is accompanied by the presence or formation of larger amounts of host specific antibody than usually met with —The host specific antibody seemed to be quite rapidly formed, pointing to anamnestic mechanisms However, the recruits had not been given vaccines grown in embryonated eggs before

The original purpose of the present investigation was to examine the frequency of heterologous HI-antibody increase resulting from A_2 vaccination It was found that a significant increase of virus specific, heterologous antibodies seldom or never occurred under the experimental conditions, which in particular may be characterized by the high percentage, 83, of vaccinees (military recruits) revealing serologically a previous experience to A_2-virus

A more irregular finding was the HI-titre increase to Lee in an unvaccinated individual as this increase probably had been provoked by an A_2-infection

SUMMARY

When purified, egg grown influenza B-virus (Lee) was used in the haemagglutination inhibition test, it was found that a single, subcutaneous injection of an ordinary preparation of egg-grown influenza A_2-vaccine produced a fourfold or greater titre increase in 18 of 40 military recruits With a similar vaccine preparation 7 of 10 individuals gave this phenomenon A titre increase was not observed when normal egg allantoic fluid was added to the sera in the test Nor was the increase seen when mouse-grown Lee virus was used in the test instead of egg-grown virus Antibody formation to soluble B-antigen was not observed It was concluded that the antibody inhibiting the purified Lee virus had

RESULTS

Experiments with simian sera Table 1 presents the titers of complement fixing antibodies reacting with measles virus antigens in single or series of serum specimens from eleven Cynomolgous monkey mothers and their babies The first mentioned had been brought to this Institute *from the Philippines* During the captivity in Copenhagen where the delivery of the babies took place, they had show no signs of illness The origin of their measles antibodies, therefore, can not be further accounted for Sera were obtained *from the animals from one day up to* about 200 days after the babies' birth As can be seen in Table 1, the titers of complement fixing measles antibodies remained unchanged in sera from four of six mothers, and showed a 2-fold decrease in two during the observation period In two instances blood samples were *drawn from the babies within the first 24 hours of life In one of these* cases the titers of complement fixing measles antibodies were identical in sera from the mother and the baby, while in the other the titer was 2 fold lower in the baby's serum than in the mother's In samples collected between eight and 25 days after the babies' birth the titers were from 2- to 4 fold lower than in sera from the respective mothers

TABLE 1

Titers of Complement Fixing Antibodies Reacting with Measles Virus Antigens in Sera from eleven Cynomolgous Monkey Mothers and their Babies

No	Cynomolgous monkeys	No of days between birth and 1st Sample	Titers of C F Measles Antibodies: In 1 Sample	In Serum Samples collected about: 35 days after 1 Sample	1 days after 1 Sample	112 days after 1 Sample	[illegible] days after 1 Sample
1	Mother		16				
1	Baby	1	16				
2	Mother		64				
2	Baby	1	32				
3	Mother		128				
3	Baby	8	64				
4	Mother		128	128	64	64	64
4	Baby	15	32	4	<4	<4	<4
5	Mother		64	64	64	64	64
5	Baby	25	32	8	8	<4	<4
6	Mother		32	32	32	32	
6	Baby	32	8	4	4	<4	
7	Mother		64	64	64	64	
7	Baby	36	16	8	4	<4	
8	Mother		64	64	32		32
8	Baby	38	8	4	<4		<4
9	Mother		64	64	64	64	64
9	Baby	46	8	4	<4	<4	<4
10	Mother		64				
10	Baby	160	<4				
11	Mother		64				
11	Baby	165	<4				

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TITERS OF COMPLEMENT FIXING MEASLES ANTIBODIES IN SERA FROM SIMIAN AND HUMAN BABIES

By

VIGGO BECH

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In an earlier paper the development of complement fixing measles antibodies in measles patients has been described (3). The titer levels of these antibodies as measured from one to about 60 years after illness have also been reported (5, 6).

This communication presents the data obtained in complement fixation experiments, in which single or paired serum specimens collected during the first year of life from human infants were tested with measles virus antigens. The results from similar experiments with single or series of blood samples drawn from eleven Cynomolgous monkey babies and their respective mothers are also described.

MATERIALS AND METHODS

Human sera. In the experiments to be described 20 samples of blood from the umbilical cord have been examined together with single serum specimens from 89 human infants and paired serum samples from 19 infants. All the infant sera were collected during the first year of life. The majority of these blood specimens had been sent to this Institute from various pediatric hospital wards to be examined for influenza antibodies. The paired serum samples were ordinarily obtained with an interval of about ten days.

Monkey sera. Single or series of blood specimens were collected during the first year of life from eleven Cynomolgous monkey babies and their mothers. In all instances samples were obtained at the same time from mother and baby. The simian babies were born in this Institute and after birth kept isolated together with their respective mothers.

The sera were prepared and stored in the same way as earlier described (3). In all cases serum specimens from one individual were tested simultaneously in the same experiment.

Virus strain. A measles virus strain isolated in the spring of 1956 in Copenhagen from the throat of a child with measles was used for the preparation of antigens (2).

Complement fixing antigens. In all tests two antigens were employed. They had been prepared as earlier described from measles infected cell cultures of the human HeLa cancer cell line maintained in a medium with horse serum and of trypsinized monkey kidney tissue respectively (3, 4). These preparations represented about four units of antigen in the complement fixation tests in which two units of complement were employed.

Complement fixation tests. The technique has been described in earlier papers (3, 4). In the experiments with human sera dilutions from 1:2 to 1:256 were examined while in the tests with sera from the simian babies the shortage of serum made it impossible to use lower dilutions than 1:4.

From the results obtained in tests with sera from the simian babies Nos 6 7, 8, and 9, it appeared that the decrease in titer was from 4- to 8-fold during the first 32 to 46 days, while in all cases it was only 2 fold during the following 35 days The antibodies remained measurable until 50 107 days after birth Correspondingly, negative titers (= values lower than four) were obtained between the 86th and the 148th day of life and onwards

Figure 1 presents the same titers as recorded in Table 1 In the Figure they have been plotted against the age in weeks of the simian babies at which the specimen was obtained

Experiments with human sera In Figure 2 the titers of complement fixing measles antibodies in blood samples drawn from human infants are recorded Like in Figure 1 these titers have been plotted against the age in weeks of the children

The sera from new borns all derived from the umbilical cord One of these sera had a titer lower than two, while the remaining 19 sera had titers between two and 64 The greatest number—five out of 20 sera—had a titer of 16

The other sera in Figure 2 were obtained from human infants between two and 56 weeks old Single blood samples had been drawn from a number of 89, while paired specimens had been collected from 19 children, usually with an interval of about ten days As can be seen in the Figure, 18 sera out of 28 obtained between the second and the 12th week, incl, gave a positive reaction with titers ranging from two to 32 Of 43 specimens collected between the 13th and the 24th week, nine sera showed a titer between two and 16, while 34 sera had titers lower than two From the 25th to the 48th week 56 samples were obtained Out of this number only four gave positive reactions Two sera had titers of two and eight, respectively, and two sera showed a titre of 32 These two last mentioned sera both derived from the same child, a boy about ten months of age

TABLE 2

Titers of Complement Fixing Measles Antibodies in Paired Serum Samples Collected with about 10 Days Interval from 6 Human Babies within the First 10 Months of Life

No	Age at Collection of 1 Sample	Antibody Titers in	
		1 Sample	2 Sample
8701	34 days	16	8
7789	39 days	8	8
8695	51 days	8	2
9250	70 days	16	16
8958	140 days	16	8
9111	273 days	32	32

Of the 19 infants from whom blood was drawn twice, a number of 13 had titers lower than two in both samples The ages of these children

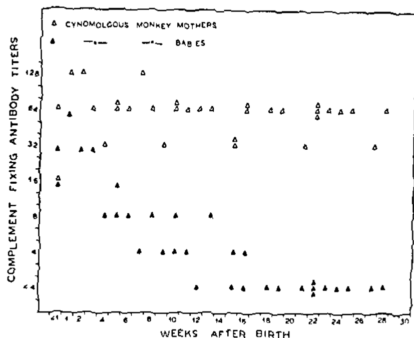


Fig 1
Titers of complement fixing antibodies reacting with measles virus antigens in sera from eleven Cynomolgous monkey mothers and their babies. Titers are plotted against the ages in weeks of the babies

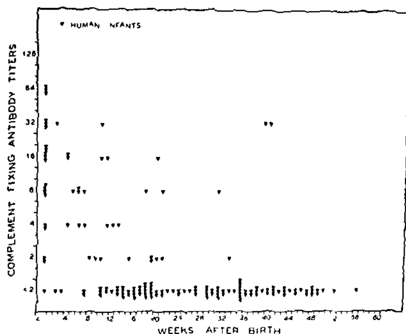


Fig 2
Titers of complement fixing measles antibodies in 20 cord blood samples and in single serum specimens from 89 human infants and in paired serum samples from 19 infants. Titers are plotted against the ages in weeks of the infants

In sera obtained during the first months of life an increasing number of negative reactions was recorded. Thus while 36 per cent of the specimens collected between the second and the 12th week after birth showed a negative reaction, 79 per cent of the sera obtained from the 13th to 24th week, and 93 per cent of samples collected between the 25th and the 48th week of life had titers lower than two. These results seem to agree well with the findings of *Karzon* (9) and *Carlström* (8) in similar studies in which they examined human sera for the content of neutralizing antibodies against canine distemper virus. This virus has later been shown to be closely related to measles virus in antigenic respect (1–7–8).

Parallel with the increasing number of negative reactions a decrease in the average titer of complement fixing measles antibodies was observed during the first months of life (see Figure 2). Only two serum samples, both obtained from a boy of about 10 months of age with an interval of about 10 days, did not fit into this general pattern. Both specimens had a titer of 32. It seems unlikely that this high antibody level should represent maternal antibodies transmitted to the infant. Probably this child, whose anamnesis unfortunately is unknown, had suffered from measles prior to the first bleeding.

Finally it should be mentioned that in this study, like in earlier experiments (4), no significant differences were encountered between the results obtained with the two different antigens employed. One of these had been prepared from measles virus infected HeLa cell cultures, the other from measles virus infected cell cultures of trypsinized monkey kidney tissue.

SUMMARY

The content of complement fixing antibodies reacting with measles virus antigens has been measured in sera collected from simian babies and human infants during the first year of life.

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were from two weeks up to 20 weeks Six of the 19 infants had titers ranging from two to 32 in the two blood samples which in these cases had been collected between the 6th and the 40th week of life The results obtained with the sera giving positive reactions are presented in Table 2, where it can be seen that the titers remained unchanged in three infants and showed a decrease of 2- to 4-fold in the other three children during the observation period of about ten days

DISCUSSION

In the experiments presented in this paper it was found that sera deriving from the eleven Cynomolgous monkey mothers all contained complement fixing antibodies reacting with measles virus antigens in titers of nearly the same magnitude (cf Fig 1)

The titer observed in each of the simian mothers showed no significant variation during the observation period which in six cases were of about 200 days' duration In sera collected from the babies of these animals an apparently uniform decrease in titers of the complement fixing measles antibodies was seen during the first months of life In two cases where blood samples were obtained within 24 hours after birth, the same or a 2-fold lower titer was recorded in sera from the babies than in sera from the respective mothers Between the 8th and the 46th day of life a difference of 2- to 8-fold was demonstrated between the titers in blood from the mothers and the babies In all instances a negative reaction (titers lower than four) was observed in the late blood samples from the simian babies In the present study the last positive reactions were recorded in specimens collected between the 50th and the 107th day of life

From the tests with sera from four of the simian babies it appeared that the decrease in titer was from 4- to 8-fold during the first 32 to 46 days, while it was only 2-fold during the following 35 days These results seem to be in accordance with observations made by *Strean et al* (13) who found that the level of neutralizing poliomyelitis antibodies in human infants gradually diminished, following roughly the shape of an exponential curve, similar to that of the excretion of a metabolite In this connection it should be mentioned that in different studies the half-life of neutralizing poliomyelitis antibodies in the new-born human infant's blood has been estimated as, respectively 21 days (11 12) 37 days (10), and 50–55 days (13)

In the experiments with sera from human infants it was observed that the distribution of titers of complement fixing measles antibodies in blood samples from the umbilical cord was very similar to that found in sera from healthy persons between one and 20 years after measles (6) Thus, only one specimen showed a negative reaction (a titer lower than two), while 19 sera had titers ranging from two to 64 the titer of 16 being the most frequent (five sera)

THE ENTEROVIRUS DEPARTMENT, STATENS SERUMINSTITUT, COPENHAGEN AND THE EPIDEMIOLOGICAL DEPARTMENT, LANDSHOSPITALET IN SONDERBORG

A CASE OF MILD PARALYTIC DISEASE DUE TO ECHO VIRUS TYPE 11

By

ANNELISE GODTFREDSEN and BENT HANSEN

Received 2 iii 61

During the past years several enteroviruses other than the three types of poliovirus have been associated with paralytic disease and evidence for the paralytogenic properties of certain serotypes of Coxsackie and echoviruses has been presented (1-11)

Recently, *Steigman & Lipton* reported that echo virus type 11 had been isolated from a stool specimen from a fatal case of bulbospinal paralytic poliomyelitis (12) No laboratory evidence of a poliovirus infection was found whereas a rise in neutralizing antibody titer to echo virus type 11 could be demonstrated

The present report contains further evidence that paralytic illness may be caused by echo virus type 11 and describes the isolation of this type of virus from the cerebrospinal fluid (CSF) of a child with clinical signs of mild paralytic polio The child had received 3 injections of formalin inactivated poliovaccine the last injection being given in August 1956

CASE REPORT

On October 5 1960 a 12 year old girl (case 7001) developed abdominal pain Severe headache appeared on the third day of illness and she vomited several times She was admitted to the hospital (Landshospitalet i Sønderborg) on the fourth day of illness

At the time of hospital admission she appeared drowsy Her temperature was 38.5° C She had marked nuchal and back rigidity and back and hamstring spasm No evidence of muscular weakness or reflex changes was found

On the day after admission moderate weakness of both arms and a corresponding diminution of deep reflexes was found Two days later she was afebrile and much improved On the 14th day after admission to hospital she complained of severe *headache and "heavyness" of the right arm* Marked muscular weakness of the right arm was noted The reflexes were normal She was discharged without residua on November 5 1960

On admission examination of CSF revealed 52 white blood cells per cubic millimeter predominantly lymphocytes Protein content 65 mg%

Laboratory Examinations

Three stool specimens collected on day 5 8 and 11 after onset of illnes one CSF taken on day 4 as well as two blood specimens were submitted to the laboratory

The technique employed in our laboratory for virus isolation virus identification

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thus be excluded that the different results obtained in the two tissue culture systems (i.e. monkey kidney cells and human amnion cells) might be due to the presence of either two viruses in the spinal fluid or to pick up of a simian virus from the monkey kidney cells employed.

Virus isolated in human amniotic cells from each of the three stool specimens could likewise all be identified in neutralization tests as echo virus type 11.

Neutralization Tests

Acute and convalescent sera collected from patient 7001 on days 4 and 18, respectively, after onset of illness were tested for neutralizing antibodies to poliovirus types 1, 2 and 3 and to the prototype strain of echo virus type 11. The results are presented in Table 1 together with a summary of the virus isolation experiments.

As will be seen no significant changes in neutralizing antibody titers to the three types of poliovirus could be demonstrated. The antibody titer against echo virus type 11 was found to increase from 2 to 11.

TABLE 1

Results of Laboratory Examination of Case 7001

Virus isolations				Serum antibodies				
Days after onset	Specimen	Results of tained in cultures of		Day of blood	Neutralizing antibody titer			
		Human amnion	Monkey kidney		Polio 1	Polio 2	Polio 3	Echo 11
4	CSF	Echo 11	Echo 11	4	1024	1024	180	2
5	Stool	Echo 11	negative	18	2048	2048	360	11
8	Stool	Echo 11	negative					
11	Stool	Echo 11	negative					

DISCUSSION

During 1960 only seven cases of paralytic polio have been reported in Denmark. In two cases the preliminary diagnosis was not maintained, the final clinical diagnosis being Guillain-Barre syndrome and paresis hypokalaemica idiopatica, respectively. From 4 of the remaining 5 patients poliovirus type 1 was isolated from the stools, one of these also contained Coxsackie B 5 virus.

Clinical and laboratory data concerning the fifth of the patients with a clinical picture of mild paralytic polio are presented in the present paper (case 7001).

The isolation of echo 11 virus from the CSF as well as from the stools together with the homologous rise in neutralizing antibody titer and the failure to isolate poliovirus from the patient justifies the conclusion that the paralytic illness of the patient was actually caused by echo 11

and neutralization tests in trypsinized monkey kidney tissue cultures has been described previously (13).

In the present study human amnion cell cultures were also employed. For preparation of this type of tissue cultures the method described by Zahelli (14) was used with some modifications. As maintenance medium for both types of cultures bovine amniotic fluid was employed.

RESULTS OF VIRUS ISOLATION EXPERIMENTS

Spinal fluid. Primary inoculation of human amnion cell cultures with CSF yielded a cytopathogenic agent, complete degeneration of the cells in 3 out of 5 inoculated tissue culture tubes being evident 9 days after inoculation. In monkey kidney cultures inoculated with CSF no degeneration of the cells was found by reading of the tubes 9 days after inoculation. However, 14 days after inoculation very slight degeneration of the cells in 3 out of 5 tubes was observed also in this type of tissue cultures. By subinoculation in monkey kidney cultures cytopathic activity became apparent more readily, complete degeneration of the cells in 5 out of 5 tubes being evident 5 days after inoculation.

Stool Specimens

Examination of the stool suspensions was carried out by parallel inoculation into monkey kidney cultures and human amnion cultures. No degeneration of the monkey kidney cells was found by the 14th day reading of the tubes and blind passage carried out in fresh monkey kidney cultures gave a negative result. In contract, in human amnion cultures complete degeneration of all inoculated tubes was evident 10 days after inoculation.

Typing Experiments

The virus strain recovered in human amnion cell cultures from CSF (Strain 7001 S) was studied in neutralization tests in monkey kidney cultures. Polio antisera types I–III and Coxsackie antisera types A9 and B1–B5 as well as monkey hyperimmune sera against echo types 1–19 were employed in the tests. The echo antisera were supplied by the courtesy of the National Foundation, USA. A final serum dilution representing at least 20 units of antibody was used in the typing experiments. The tests showed that 600 and 6000 TCD_{50} of virus strain 7001 S were neutralized by echo antiserum type 11 only.

The result was verified by re-testing strain 7001 S together with the prototype strain of echo 11 in a neutralization test against echo antiserum type 11. It was found that 10 000 TCD_{50} of strain 7001 S and 40 000 TCD_{50} of the prototype echo 11 strain were completely neutralized by echo antiserum type 11.

The agent recovered from CSF by inoculation of monkey kidney cells was likewise identified in neutralization tests as echo 11 virus. It could

previously examined in monkey kidney cultures with negative result were re-tested by inoculation in human amnion cultures. None of these gave positive results.

TABLE 2

Cell Spectrum for Echo 11 Virus as Reported by Various Investigators and for Strains 7001 and 7001 S

Virus isolated by	Reference no.	Monkey kidney cells: primary	Monkey kidney cells: passage	Human embryonic lung	HeLa	Detroit	Maben	Human amnion
Ramos Alvarez & Sabin 1954	15	+						
Ormsbee & Melnick 1957	16	+			+		+	
Ramos Alvarez & Sabin 1958	17	+						
Philipson & Wesslen 1958	18		±	+	—	—		
Elvin Lewis & Melnick 1959	20	+						
von Zeipel et al. 1960	21	+			+			
Steigman & Lipton 1960	12	+	—		—	—		
Strain 7001*		—						+
Strain 7001 S§		(+)						+

+ = cytopathogenic effect
± = adaptation to monkey kidney cells (cytopathogenic effect) obtained after 5 passages
(+) = slight cytopathogenic effect on monkey kidney cells
* virus isolated from stool specimen from case 7001
§ virus isolated from CSF from case 7001

SUMMARY

Laboratory studies on a case of mild paralytic disease in a 12 year old girl are presented. The child had received a series of 3 polio vaccinations, the last given in 1956.

Echo virus type 11 was recovered from CSF as well as from three stool specimens and a rise in homologous antibody level was demonstrated in paired serum samples. No evidence of infection with polio virus could be demonstrated.

It is concluded that the paralytic illness was caused by echo virus type 11.

Virus was recovered from CSF more readily in human amnion cell cultures than in monkey kidney cultures. Three stool specimens all yielded echo virus type 11 by inoculation in human amnion cultures whereas inoculation of monkey kidney cultures gave negative results.

Strains of echo virus type 11 have been isolated previously from normal children (15, 16) from sporadic cases of diarrhea (17) and from patients suffering from upper respiratory diseases (18, 19) Other workers recently observed the acute meningitis syndrome to be associated with echo 11 virus infection (20, 21) and evidence for the capacity of this type of virus to produce more serious neurolytic damage has been presented by *Steigman & Lipton* (12)

So far, monkey kidney cultures have been considered to be one of the best indicators for the presence of echo viruses in clinical specimens, and the cytopathogenicity for this cell type is actually considered a characteristic of the echo viruses For several years this type of tissue cultures have been used in our laboratory for isolation of polio- and echoviruses (13)

In May-December 1960 the majority of cerebrospinal fluids received in our laboratory have been examined by parallel inoculation in 5 monkey kidney tissue cultures and 5 human amnion tissue cultures

During this period virus was recovered from 32 out of 423 cerebrospinal fluids examined

A comparison of the susceptibility of the two types of tissue cultures is rendered difficult by the fact that in many cases cell degeneration was found only in 1 or 2 of the 10 tissue culture tubes inoculated with cerebrospinal fluid In such instances it cannot be said whether the different response in the two types of tissue cultures represent different cell susceptibility

It should be mentioned, however, that 17 cerebrospinal fluids which were found to contain Coxsackie B virus (15 B5, 1 B2 and 1 B4) all gave positive results by inoculation in monkey kidney cultures whereas only 3 of these specimens gave positive results also by inoculation of human amnion cell cultures It seems possible therefore that for isolation of Coxsackie B-5 strains monkey kidney cells are superior to human amnion cells

In the case reported here echo 11 virus was recovered from CSF more readily in human amnion cell cultures than in monkey kidney cultures Attempts to isolate virus from the stool specimens in monkey kidney cells were completely negative, whereas echo virus type 11 was isolated readily from all three specimens by inoculation in human amnion cell cultures

The types of tissue cultures employed in earlier reports on isolations of echo 11 strains are summarized in Table 2 With the exception of the echo 11 strain isolated by *Philipson* (18) all strains were isolated by inoculation of monkey kidney cells It seems possible therefore that the echo 11 strain 7001 described in the present paper represent a rather rare cell specificity

In order to examine whether we might have missed some echo 11 strains by using MKC only in our attempts to isolate viral agents from stools, 28 fecal specimens received during October-November 1960 and

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TWO NEW STRAINS OF PASTEURELLA HAEMOLYTICA VAR. UREAE ISOLATED FROM THE RESPIRATORY TRACT

By

TOVE OMLAND and SVERRE DICK HENRIKSEN

Received 7.iii.61

As judged from previous literature organisms belonging to the genus Pasteurella have only been isolated infrequently from the human respiratory tract. However in a recent paper the finding of altogether 10 Pasteurella strains from different parts of the upper respiratory tract was described (2). This also contains a review of the pertinent literature.

Especially interesting is the isolation of 4 strains of a new variety for which the designation Pasteurella haemolytica var. ureae was proposed (1). The special characteristics of this variant are: 1) The appearance on microscopical examination of pleomorphic coccobacilli to filaments 0.5–0.7 microns wide. 2) The appearance on blood agar of a marked green discoloration, usually accompanied by a partial haemolysis. 3) Colonies of a slightly translucent and mucoid appearance, the diameter being about 2 mm after 24 hours of incubation. 4) Fastidiousness in its nutritional requirements (serum, ascitic fluid etc.). 5) Comparatively [illegible] ing. 6) [illegible] ture o[illegible] pathog[illegible] ordinary laboratory animals (mice, guinea pigs, rabbits) in moderate doses.

The main properties separating the mentioned variety from Pasteurella haemolytica are: a very strong urease activity, failure to ferment lactose and xylose, unwillingness to grow on simple media.

The purpose of the present report is to describe another two strains showing the same characteristics as those previously described (1, 2) under the name Pasteurella haemolytica var. ureae.

MATERIAL

Strain KA 1597/60, isolated from a 49 year old man admitted to Surgical department A under the diagnosis tumor pulmonis. The organism was found in his sputum, the finding being however apparently unrelated to his clinical condition. Only one

These echo 11 virus strains were thus characterized by higher affinity for human amniotic cell cultures than for monkey kidney cultures, and accordingly represent an unusual variety amongst echo strains

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towards uneven staining, and some of the long forms had oval "vacuoles"

Growth The strains conformed with the characteristics mentioned in the introduction Especially marked was their unwillingness to grow in simple media (*e g* peptone water without sugar)

Biochemical properties These were the same as previously reported, with the exception that one of the strains (585 61) produced traces of acid from sorbitol and xylose The media for the indole reaction, the methyl red and Voges Proskauer reactions and the nitrate reaction had to be enriched by horse serum (5 per cent) The urease reaction was extremely strong (visible after few minutes, complete after 2 hours)

Sensitivity to chemotherapeutics and antibiotics The results are shown in Table 1 The two strains have a practically identical pattern which is in good conformance with previous findings (2)

TABLE 2

Pathogenicity of 2 Strains of Pasteurella haemolytica var ureae (Identical Results for the two Strains)

Mouse (2 animals per dose)

Dose (intraperit)	Time after injection 24 h	48 h	72 h
0 5 ml	2 dead		
0 05 ml	0 dead	1 dead	
0 005 ml	0 dead	0 dead	0 dead

Guinea pig (2 animals)

Dose 0 5 ml subcutaneously
Slight transient local lymph node enlargement, otherwise unaffected

Rabbit (1 animal)

Dose 0 5 ml intravenously
Toxic effect during the first 1 2 days (anorexia weakness) otherwise unaffected

Pathogenicity The results of our animal experiments are recorded in Table 2 The effect on mice of high doses must be considered of a toxic nature Apart from this the organism appeared to have a very low degree of pathogenicity Too little evidence is as yet available to get any definite impression of the pathogenicity to man The isolation of one of the strains in pure culture (585 61) from a pathological condition (sinusitis) points towards a certain pathogenecity

SUMMARY

The isolation from the human respiratory tract of two new strains of Pasteurella haemolytica var ureae is reported

The isolated strains possessed a very low degree of pathogenicity in

specimen of sputum was submitted for ordinary bacteriological examination during the patient's stay in hospital and consequently nothing can be said as to the duration of the "carrier state". The sputum yielded an almost pure growth of the organism.

Strain 585/61 isolated from a 60 year old man suffering from sinusitis maxillaris. The aspirated pus showed on direct microscopy abundant masses of polymorphonuclear leucocytes and small groups of somewhat pleomorphic Gram negative rods with slightly uneven distribution of the stain, partly also with uncolored "vacuoles". The material yielded a pure culture of the organism.

METHODS

The methods were mainly the same as those described in a previous paper (2). The oxidase reaction was performed by means of tetramethyl-p-phenylenediamine reagent only.

The pathogenicity was examined by injection on mice, guinea pigs and rabbits of a 24 hour culture on blood agar. The organisms were suspended in saline and standardized photometrically to an opacity corresponding to about 1500 millions staphylococci per ml. The mice received the injections intraperitoneally, the guinea pigs subcutaneously and the rabbits intravenously.

RESULTS

Morphology. Both strains yielded mucoid, partly translucent colonies of up to 2 mm diameter on blood agar after 24 hours of incubation at 37° C. The colonies were surrounded by a zone of distinct greenish discoloration, however without a regular beta-haemolysis. This phenomenon was reproducible both in surface cultures and in poured plates. The discoloration zones became especially distinct after some days on the desk.

TABLE 1

Sensitivity to Chemotherapeutics and Antibiotics of 2 Strains of Pasteurella haemolytica var. ureae

	Strains	
	KA 1427/60	585/61
Sulphonamide	1 (44)	1 (45)
Penicillin	2 (21)	3 (20)
Streptomycin	1 (25)	1 (25)
Chloramphenicol	1 (31)	1 (32)
Chlortetracycline	1 (20)	1 (22)
Oxitetracycline	1 (22)	1 (23)
Tetracycline	2 (16)	2 (18)
Erythromycin	1 (30)	1 (30)
Oleandomycin	3 (18)	3 (19)
Novobiocin	4 (9)	4 (10)
Kanamycin	3 (17)	3 (18)

1 fully sensitive, 2 moderately sensitive, 3 relatively resistant, 4 fully resistant. Numbers in brackets represent diameters of inhibition zones.

Microscopical examination showed pleomorphic Gram-negative rods with rounded sides, partly with bipolar staining. The width of the cells was fairly constant (0.5-0.7 micron), but the length varied from short coccobacilli to long filaments. On the whole there was a tendency

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LYSIS OF BACTERIA

3. On the Stability of Protoplasts and Spheroplasts in Different pH Ranges[1]

By

LARS LDEBO

Received 21 iii 1961

When bacterial suspensions are subjected to sonic oscillation at neutral or alkaline pH they usually lyse. This is due to a disintegration of the cell wall which is accompanied by dispersion of protoplasm. When both the disintegration and the dispersion occur the result is lysis. If the bacterial suspensions are sonic treated at pH values below 5.0–5.5 where the solubility of the protoplasm is decreased the sonic lysis is diminished although the disintegration of the cell walls is not conspicuously affected (*Edebo* 1961 a, b). Sonic treated cells, protoplasts and spheroplasts resemble each other in that each partially or completely lacks the protecting and supporting effect of the cell walls which usually withstand the high intracellular pressure. Thus if protoplasts and spheroplasts are suspended in neutral solutions with low osmotic pressure they burst, their suspensions lyse and the protoplast content is dispersed (*Weibull* 1953). Since the lysis of sonic treated suspensions is inhibited at conditions which reduce the solubility of the protoplasm, i.e. at pH values below 5.0–5.5, investigation of the behavior of protoplasts and spheroplasts under similar conditions at low osmotic pressure is of considerable interest.

MATERIALS AND METHODS

Bacillus megaterium, strain KM* [illegible] 15–4 hours at 37° C. After growth [illegible] twice in 0.06 M potassium phosphat[illegible] hagen). Half the mass of the cells w[illegible] 0.2 M sucrose, 0.04 M potassium pho[illegible] magnesium ions were added. In ord[illegible] plasts (*C. Weibull* 1956). The remaining half of the cellular material was frozen and stored at −25° C for later disintegration.

This work was made possible by a grant from the *Wallenberg Foundation*. The technical assistance provided by Miss *Lilla Spet* is gratefully acknowledged.

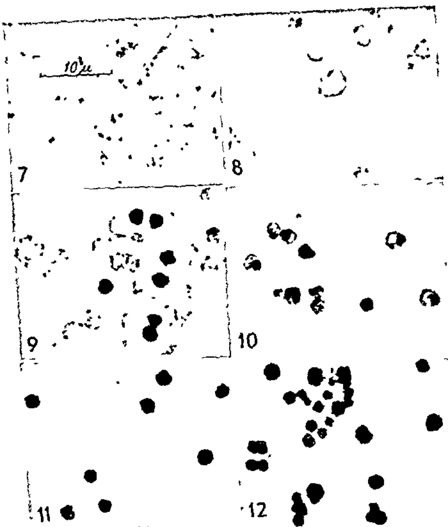
* Kindly supplied by Dr. *C. Weibull*.

[1] Originally presented at a meeting of the Swedish Medical Society, December 1959.

animal experiments One of the strains was isolated in pure culture from a pathological condition (sinusitis) The question of its pathogenicity towards man needs further clarification

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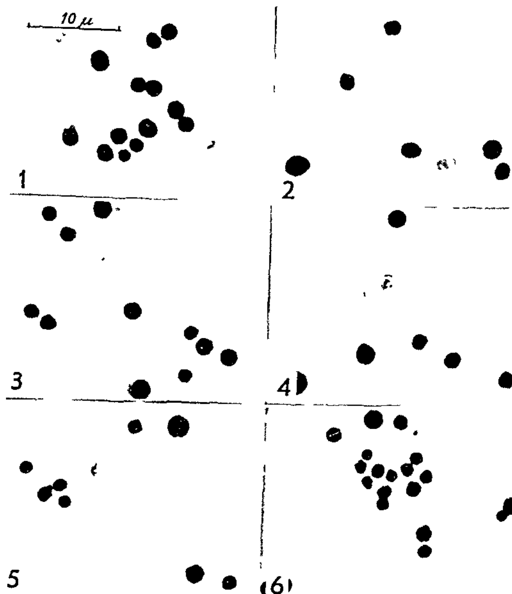


for 20 min and preparation of the sediments for phase contrast and electron microscopy followed. These photographs were completed within 6 hours.

Spheroplasts from *Escherichia coli B* were *prepared by the lysozyme versene* method (*Repaske* 1958). The spheroplasts were washed and suspended in 0.9 per cent [illegible] rate cellophane tubings and dialysed [illegible] had been adjusted to pH 4, 4.5, 5 and [illegible] *After dialysis at +3° C* for 17 hours, [illegible] and electron microscopy and smears made for staining with dilute fuchsin. *For fixation* a portion of each suspensions was adjusted to 1 per cent with osmium tetraoxide.

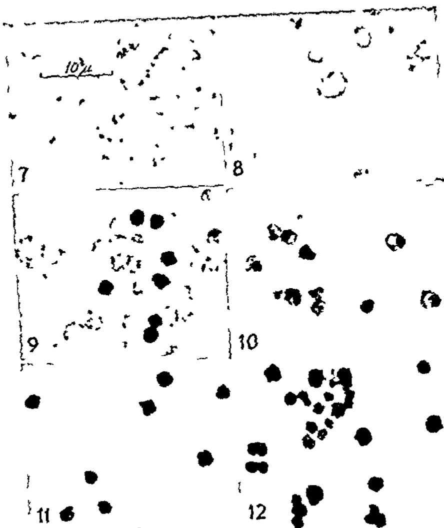
RESULTS

When the pH of the *protoplast* suspension was reduced to values below pH 5, the protoplasts shrank and showed some tendency to ag-



Lysozyme was added to the bacterial suspension to a final concentration of 0.1 mg/ml and the tubes were placed in an ice water bath for 3 hours. Protoplasts prepared in this fashion can be maintained for some days in the refrigerator without bursting. When the suspension consisted entirely of spherical protoplasts it was divided into equal (3 ml) samples. Each was adjusted to a different pH value by adding 5 drops of appropriate mixtures of 1 M phosphoric acid and 1 M potassium dihydrogenphosphate from a Pasteur pipette and left in an ice water bath. Specimens for phase contrast photomicrography (Zeiss Photomikroskop) were prepared within 3 hours from these samples by placing a very small drop on a glass slide with a platinum loop and then gently lowering the cover slip onto the drop without extra pressure. Visual fields were selected where one or two protoplasts were slightly moving. The maximum time difference between samples of different pH values was 3 hours. By alternatively choosing acid and neutral samples and by running a similar experiment in another order, time effects were ruled out.

When all preparations had been photographed, the samples were diluted 1 : 10 with 0.03 M potassium phosphate solutions of similar pH, and the extinction at 600 mμ measured in a Beckman DU spectrophotometer. Centrifugation at 6 000 × g



for 20 min and preparation of the sediments for phase contrast and electron microscopy followed. These photographs were completed within 6 hours.

Spheroplasts from *Escherichia coli* B were prepared by the lysozyme versene method (*Repaske* 1958). The spheroplasts were washed and suspended in 0.9 per cent sodium chloride, poured into four separate cellophane tubings and dialysed against 200 volumes of distilled water which had been adjusted to pH 4, 4.5, 5 and 7 respectively with 3 N hydrochloric acid. After dialysis at +3° C for 17 hours, specimens were prepared for phase contrast and electron microscopy and smears made for staining with dilute fuchsin. For fixation a portion of each suspension was adjusted to 1 per cent with osmium tetraoxide.

RESULTS

When the pH of the protoplast suspension was reduced to values below pH 5 the protoplasts shrank and showed some tendency to ag



Lysozyme was added to the bacterial suspension to a final concentration of 0.1 mg/ml and the tubes were placed in an ice-water bath for 3 hours. Protoplasts prepared in this fashion can be maintained for some days in the refrigerator without bursting. When the suspension consisted entirely of spherical protoplasts, it was divided into equal (3 ml) samples. Each was adjusted to a different pH value by adding 5 drops of appropriate mixtures of 1 M phosphoric acid and 1 M potassium dihydrogenphosphate from a Pasteur pipette and left in an ice water bath. Specimens for phase contrast photomicrography (Zeiss Photomikroskop) were prepared within 3 hours from these samples by placing a very small drop on a glass slide with a platinum loop and then gently lowering the cover slip on to the drop without extra pressure. Visual fields were selected where one or two protoplasts were slightly moving. The maximum time difference between samples of different pH values was 3 hours. By alternatively choosing acid and neutral samples and by running a similar experiment in another order, time effects were ruled out.

When all preparations had been photographed, the samples were diluted 1:10 with 0.03 M potassium phosphate solutions of similar pH and the extinction at 600 mμ measured in a Beckman DU spectrophotometer. Centrifugation at 6000 × g

gregate (Fig 5, 6) In this pH-range neither did they burst on dilution to low osmotic pressures (Fig 11, 12), nor did the optical density (extinction at 600 mμ) of the suspensions disappear At pH values between this range and neutrality, the behavior of the protoplasts was intermediate At pH 5 5 (Fig 9) some protoplasts burst while part were preserved, and at pH 5 3 (Fig 10), the protoplasts did not appear to burst, but showed bud like extrusions At pH 6 3, the protoplasts burst leaving only sparse plasma membranes behind (Fig 7) while at pH 6 0 the plasma membranes were better preserved (Fig 8) The optical density readings showed a smooth course of lower values as the pH increased and correlated well with the microscopical observations The conditions inhibiting the burst seemed to be largely reversible (Exp 7, Fig 13) When protoplasts in neutral suspension were diluted by acid buffers a few protoplasts were preserved but most burst (Fig 15) The electron microscopical observations agreed with these results

TABLE 1
Protoplasts at Different pH Values

Experiment nr	pH			Extinction 600 mμ	pH After centrif	Figures	
	Before dilution	Dilution fluid	After dilution			Before dilution	After dilution
1	6 4	6 4	6 3	020	6 3	1	7
2	5 8	5 8	5 9	020	6 0	2	8
3	5 5	5 6	5 6	115	5 5	3	9
4	5 2	5 2	5 3	205	5 3	4	10
5	4 7	4 8	4 9	.335	5 0	5	11
6	4 3	4 4	4 4	395	4 4	6	12
7	4 3	7	6 5	080	6 6	6	13
8	6 4	4	5 5	045	5 5	1	14
9	6 4	3.2	4 9	125	4 9	1	15

Protoplasts prepared in absence of magnesium ions also resisted osmotic lysis at low pH values, and protoplasts suspended in acid sucrose gained in stability

The behavior of E coli spheroplasts, which were similarly manipulated, was not equally clear-cut If observed by the [illegible] phase contrast and in electron microscopic and fuchsin-stained specimens, the spheroplasts which were dialysed to pH values below 5 0 5 5 (Fig 16, pH 5 3) were less disintegrated than spheroplasts at higher pH (Fig 17, pH 7 2) Again the optical density was higher in the samples of lower pH

10 μ

13

14

15

Explanation of Figs. 1-15, see Table 1

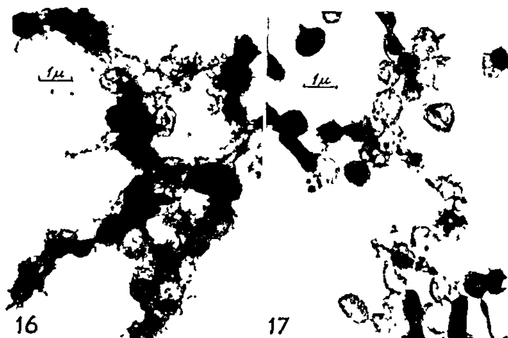


Fig. 16
Spheroplasts from E. coli dialysed to pH 5.3

Fig. 17
Spheroplasts from E. coli dialysed to pH 7.2

(cf *Edebo* 1961) However, it should be remembered that also the plasma membrane is a gel, as are all natural semipermeable membranes (*Jirgensons* 1958), and its colloidal state is also affected by the surrounding conditions. Thus, magnesium ions have been used for the stabilization of the plasma membrane (*Weibull* 1956, *McQuillen* 1960), and from the present investigation (Figs 7–12) it is evident that the plasma membrane is better preserved at lower pH values.

In future experiments it is planned to test osmotically fragile, reproducing bacteria for survival when artificially stabilized in solutions of low osmotic pressure.

SUMMARY

Protoplasts from Bacillus megaterium were more stable in the acid pH range. Below pH 5 protoplasts did not burst when subjected to osmotic shock. At intermediate pH values it was usual to find either that only a few protoplasts remained intact or that bud like extrusions appeared and complete bursting did not occur.

Spheroplasts from E. coli behaved in a similar way. It is postulated that the inhibition of osmotic bursting of protoplasts and a similar phenomenon, the inhibition of sonic lysis of bacteria, are favoured by conditions which reduce the solubility of cellular constituents.

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DISCUSSION

In 1953, *Weibull* presented the definitive work which established the protoplasts as the osmotically fragile protoplasmic unit of the bacterium Over the years prior to the Weibull studies, however, several agents were found which inhibited the lysis of bacteria by lysozyme As early as 30 years ago it was established that agents which impaired the solubility of the protoplasm also inhibited lysis, *e g* increased hydrogen ion concentration and heat treatment (*Hallauer* 1929, cf *Salton* 1957), copper ions (*Andersen* 1931), organic solvents and iodine (*Epstein & Chain* 1940), and formaldehyde (*Welshimer & Robinow* 1949) Cations were arranged according to their inhibiting effect on lysis (*Boasson* 1938, *Smolelis & Hartsell* 1952; *Grula & Hartsell* 1957), *viz*, Al > Zn > Ca > Mg, Ba > NH_4 > K > Na Polycations such as polymyxin (*Newton* 1955) and streptomycin (*Welsch* 1955) inhibited lysozyme lysis of bacteria Similarly, osmotic lysis of E coli "protoplasts" was prevented by spermine (*Mager* 1959), and spermine and spermidine stabilized mitochondria and "protoplasts" (*Tabor* 1960)

In 1923 *Nakamura* reported that bacteria originally insensitive to lysozyme lysed if they were treated with lysozyme at acid pH (pH 3 5) and then made alkaline (pH 10) *Grula & Hartsell* (1957) demonstrated that this was probably due to the uncovering of the lysozyme substrate by acid treatment, which made digestion of cell wall possible However, they found alkaline rather than acid conditions were favourable for dispersion of the protoplasm, and addition of alkali was indeed required for the second step, the lysis

The inhibition of lysozyme lysis of bacteria and the osmotic lysis of protoplasts under conditions just outlined is remarkably similar to the impairment of sonic lysis reported by *Edebo* (1961) In the present investigation, the bursting of protoplasts and sonic lysis were done with bacteria from the same culture Both were inhibited in a range around pH 5 It is postulated that these two reactions are due to a similar mechanism, *i e*, one which depends on the solubility of the protoplasm

Long ago it was observed that when nucleic acids and basic proteins were mixed, a precipitation occurred (*Miescher* 1897) When a protein, such as albumin, whose isoelectric point is in the acid range, is mixed with nucleic acid at neutral pH, a stable solution occurs However, a precipitation is elicited if pH is decreased (*Hammarsten* 1924 *Hammarsten et al* 1928) It is probable that such a nucleic acid— protein reaction also occurred in the protoplasts Under conditions favouring a precipitation, the transformation of a gel into a colloidal solution is inhibited (*Jirgensons* 1958), *i e*, the dispersion of the protoplasmic gel of the protoplasts into the surrounding medium is impaired

The inhibition of protoplast lysis by increased hydrogen ion concentration, polycations and polyvalent cations (Hofmeister series) may be entirely explained by their reduction of the solubility of the protoplasm

(cf *Edebo* 1961) However, it should be remembered that also the plasma membrane is a gel, as are all natural semipermeable membranes (*Jirgensons* 1958), and its colloidal state is also affected by the surrounding conditions Thus, magnesium ions have been used for the stabilization of the plasma membrane (*Weibull* 1956, *McQuillen* 1960), and from the present investigation (Figs 7–12) it is evident that the plasma membrane is better preserved at lower pH values

In future experiments it is planned to test osmotically fragile, reproducing bacteria for survival when artificially stabilized in solutions of low osmotic pressure

SUMMARY

Protoplasts from Bacillus megaterium were more stable in the acid pH range Below pH 5 protoplasts did not burst when subjected to osmotic shock At intermediate pH values it was usual to find either that only a few protoplasts remained intact, or that bud like extrusions appeared and complete bursting did not occur

Spheroplasts from E coli behaved in a similar way It is postulated that the inhibition of osmotic bursting of protoplasts and a similar phenomenon the inhibition of sonic lysis of bacteria, are favoured by conditions which reduce the solubility of cellular constituents

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LONG TERM CYCLIC VARIATIONS IN THE ELECTRICAL BEHAVIOR OF HEART TISSUE IN CULTURE

By

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This report concerns the average electrical behavior of 107 whole heart explants from 7-day chick embryos kept alive for 6 days in artificial conditions outside the body. It provides data to amplify and interpret more fully, the cyclic variations in the electrical behavior of heart tissue in culture seen in a previous similar study (*Cunningham & Ekborn* 1960). This previous study will be referred to as study 1 and the material presented in this paper as study 2. Study 2 is based on more than 150,000 fifteen second surveillances involving over four million observations. A search of the literature has failed to reveal any comparable publication save that of our own previous study.

TECHNIQUES

107 whole 7-day chick embryo hearts in three separate similar groups were used in the present study. The first involved 38, the second 33 and the third 36 heart explants. Each heart was treated in an identical fashion [illegible]

[illegible] flow of

[illegible] each amplified signal from each explant caused a constant width, constant am-

This investigation was supported by a USPHS *Research Grant (H 2249) from the National Heart Institute, U.S. Public Health Service.*

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plitude signal to be generated in a pulse counter generator Using circuitry similar to that for measuring the magnitude of the potentials the outputs of the counter generators is fed through a mixer, an operational rate integrator, to a computer and the average frequency of potential production per active culture per 15 second period is obtained Both the readouts of the average potential per active heart per 15 seconds and the average frequency of potential production per active heart per fifteen seconds are obtained simultaneously as straight lines on a dual channel strip chart recorder, both being of the type seen in Figure 1 Five seconds silence is allowed between each survey so that the pens of the strip chart recorder can return to zero

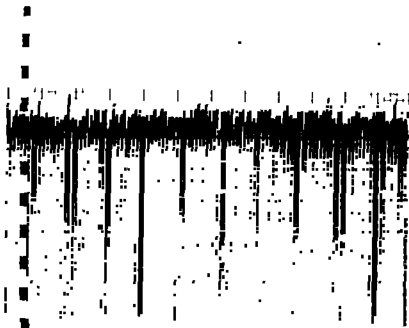


Fig 1
The type of trace produced by DINAH for both the average potential and the average contractile rate per active heart per fifteen seconds Each vertical stroke is a linear representation of the variable involved The whole sequence represents a half hours activity

Temperature

The temperature in the incubators containing the cultures was carefully controlled and did not vary by more than 0 2° C from 37° C save for short periods every day when the incubators were opened for inspection of the cultures However, this time of inspection varied from day to day and did not coinside with any variations in the rate or potential for any of the three groups of cultures

Differences Between Studies 1 and 2

Many of the differences in experimental technique and materials between this study and the previous one are given in Table 1 Other differences between the two studies need a fuller explanation and are

(1) Supernatant, that used for study 1 was a 0 25 per cent solution of human serum protein in Hanks' balanced salt solution with a change after 24 hours to Synthetic Supernatant 199 (DIFCO) The supernatant used throughout the second study was a 0 25 per cent solution of human serum protein in balanced salt solution TDLI (*Cunningham, Doughterty & Rylander* 1960)

TABLE 1

	This study	Previous study
No. of cultures	107	61
Age of embryos	7 days	6 days
Frequency of observation of potentials	for 15 out of every 20 consecutive seconds	one potential from each heart every fifty min
Frequency of observation of rate	for 15 out of every 20 seconds	averaged overall every 100 minutes
Minimum potential recorded as a pulse in the rate recorders	0.0 mv	0.02 mv
Culture technique	effectively the same	effectively the same
Temperature	closely controlled	some variation
Reference areas whose average is the 100 per cent value of rate	87½–104 hrs in culture	whole life
Reference areas whose average is the 100 per cent value of potential	87½–100 hrs in culture	100–116½ hrs
No. of observations	over 5 000 000	10 782

(2) Selection of the cultures for the two studies: study 1 used cultures which had maintained adequate electrical activity for at least one day longer than the period for which the data was used, the selection of the records to be used being done at the end of the study. In study 2 observations were made automatically and almost continuously on all of an unselected group of explants for the whole period for which the data was required whether or not they maintained electrical function. Thus the data in the second study arises in part from explants which were in the process of electrical failure. The actual detail of the electrical survival of the hearts in this second study have already been published (*Cunningham, Lunell & Rylander* 1961).

(3) The culture technique adopted in study 2 was the same as that used in study 1. The culture chambers and flow systems in the two studies differed in physical form but the final flow past the sponges holding the explants was the same in both studies.

(4) All the explants in study 2 were made within an hour of 9 a.m. whereas half of the explants in study 1 were made within an hour of 10 a.m. and the other half within an hour of 3 p.m.

RESULTS AND DISCUSSION

Electrical Potential

The vertical height of each individual peak of the read-out for potentials from DINAH from the baseline was a linear representation of the average potential per electrically active culture for the previous fifteen seconds. The peaks of the 15 second surveys during a half hour

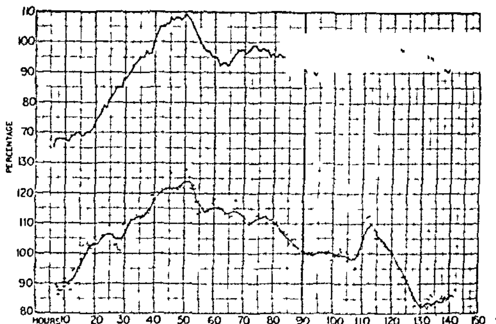


Fig 2

Upper curve, 700 minute moving average of the average potential per minute per heart in study 1 expressed as a percentage of the behavior between 100 and 116½ hours in culture Y axis—percentage potential/minute/heart X axis—time after explantation in hours

Lower Curve, Continuous line—5 hour moving average of the average potential per fifteen seconds per heart in study 2 expressed as a percentage of the behavior between 87½ and 104 hours in culture Isolated points represent the individual values from which the moving average was made Y axis—percentage potential/fifteen seconds/heart X axis time after explantation in hours

on the record from the strip chart recorder were joined and the area between this line and the baseline was measured planimetrically The value obtained represented the average of a half-hour sequence of such fifteen second surveys and similar half hour averages were measured for each half-hour in this study Half-hour averages of this type were expressed as percentages of the average activity between 87½ and 104 104 hours in culture They were then plotted against time to give a curve representing the change in average potential production by the cultures during the period of the study

In expressing this or any of the other variables (rate, etc) as proportions of the average behavior between 87½ and 104 hours in culture both the divisor and the dividend are subject to the same constants (the time constant and that governing the relationship between the planimetric half hour average and the actual half hour average) When the division is carried out and the proportion is found, these constants become unity and can be ignored

Figure 2 shows the remarkable similarity between curves of this type for studies 1and 2 This similarity confirms that the changes seen must

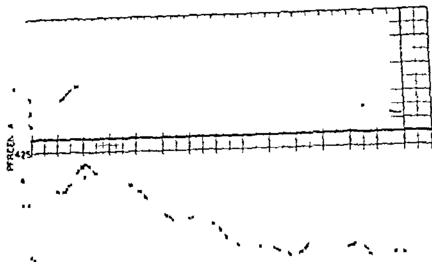


Fig 3

Upper curve 700 minute moving average of the average contractile rate per minute per heart in study 1 expressed as a percentage of the overall behavior in culture Y axis—percentage contractile rate/minute/heart X axis—time after explantation in hours

Lower Curve Continuous line—5 hour moving average of the average contractile rate per fifteen seconds per heart for study 2 expressed as a percentage of the behavior between 87 z and 104 hours in culture Isolated points represent the individual values from which the moving average was made Y axis—percentage contractile rate/15 seconds/heart X axis time after explantation in hours

represent the true reaction of heart tissue to the process of aging in culture the true trend of cardiac action in culture The differences in level of the two curves with respect to the 100 per cent mark arises because this 100 per cent level in study 1 was the average behavior from 100 116½ hours in culture which is at the top of one of the major peaks of cyclic change common to both studies and detailed later On the other hand the 100 per cent value for study 2 is the average behavior between 87½ and 104 hours in culture which lies in one of the troughs of cyclic activity and is therefore a smaller value than the 100 per cent value for study 1 The similarity between these two curves extends *to* even the minor cyclic variations and is obviously close even on visual inspection There are similar major peaks in the two curves at about 24 48 72 and 108 hours *e g* at daily intervals In study 1 we had previously attributed the cyclic variations to changes in temperature in the incubator However since the same variations are present in study 2 in which the incubators were carefully temperature controlled this conclusion must be abandoned Also since practically everything in the two experiments was different save the heart tissue and the cellulose

sponge, it now seems that this daily cyclic variation must be considered to be an inherent property of cardiac tissue in culture An examination of the curves showing the changes in average potential with the passage of time for the two studies also shows the presence of somewhat indefinite cycles The peaks of cyclic changes are 25 hours for study 1 and 17 hours for study 2

Rate of Contraction

The 15 second read-outs for the rate from DINAH are integrated as half-hour averages in the same way as for the potentials, expressed as a percentage of the average behavior between 87½ hours and 104 hours in culture and then plotted against time Comparison with the graphic representation of the same function from study 1 (Fig 3) shows that although the two curves have a definite resemblance, the rate in study 2 is distinctly more labile and runs at a distinctly higher level for the first 83 hours in culture This may be the result of any one of the differences between the two sudies—the age of the embryos, the periods chosen for the 100 per cent level (if this were corrected it would not make more than a 10 per cent increase in the values in study 1) or possibly the fact that none of the hearts in study 1 were in the process of undergoing electrical failure while some of those in study 2 were possibly in the process of undergoing electrical failure The data on the persistence of electrical potential production in 141 whole heart explants (including all those used in study 2) has already been published (*Cunningham, Lunell & Rylander* 1961) It shows that between 75 and 91½ hours in culture the electrical persistence curve flattens out and the 'electrical failure' rate drops presumably together with the concentration of the agents that cause the failure The levelling out of both the rate and potential curves for study 2 during the same time in culture supports the idea that the initial high level of activity may have been a reaction to 'tissue breakdown products' resulting from damage during explantation However, after this time (91½ hours) changes in the rate and potential curves seem to bear no relation to those in the survival curve

The presence of a cyclic variation in the rate curves for both studies in spite of exacting temperature control in study 2 again seems to confirm the cyclic activity as an innate property of heart tissue in culture

After the second day, the similarity of the rate and the potential curves in the two studies suggests that the occurrence of electrical failure in some of the cultures under surveillance in study 2 has made little difference to the overall average behavior of the hearts in this study Thus, the continuous monitoring of a predetermined number of hearts including those that fail to produce electrical discharges for the whole period of the study gives similar results to studies in which all

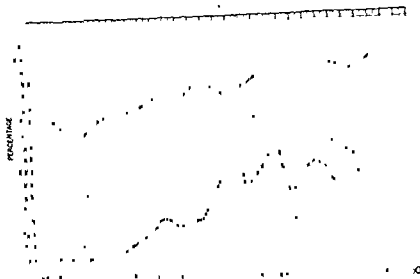


Fig 4

Upper curve, 700 minute moving average of the average potential per beat in study 1 expressed as a percentage of the average behavior of the hearts in this study in this respect Y axis—potential per beat X axis—time since explantation in hours

Lower Curve 5 hour moving average of the potential per beat for study 2 expressed as a percentage of the average behavior between 87½ and 104 hours in culture Y axis—potential per beat X axis—time since explantation in hours

of the hearts produced potentials all of the time It is also a more practical and economical method The cyclic change in the rate curve can be seen in Figure 3 It has an average interval of 20 hours for study 1 and 17 hours for study 2

Average Potential per Contraction

The values for the average potential per contraction were obtained in both studies by dividing the percentage average potential for a half-hour by the percentage average rate for the same half-hour The similarity of the trend and cyclic changes in the two studies are evident (Fig 4) and act as mutual corrobaration The values for study 2 start at a lower level because of the higher initial rate in this study but the eventual trends in the two studies are the same The values for study 2 are more labile probably because of the greater number of observations and the shorter duration of the moving average allowing a more detailed look at the behavior The average interval between the peaks of the cyclic change are 15½ hours for study 1 and 17 hours for study 2

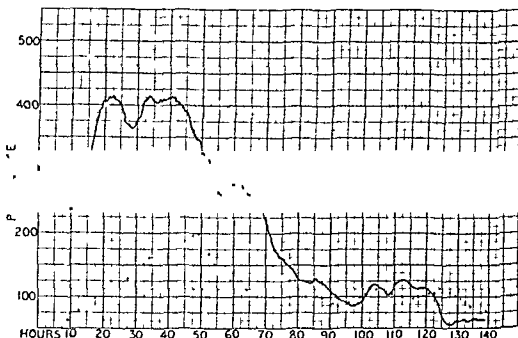


Fig 5

Solid line—5 hour running average of the average expenditure of energy in the production of electrical potentials in study 2 expressed as a percentage of the average behavior between 87½ and 104 hours in culture

Dotted line—700 minute moving average of the average expenditure of energy in the formation of electrical impulses for study 1 expressed as a percentage of the average behavior of the hearts in this study in this respect

X axis Time in hours since explantation Y axis Percentage energy expenditure in the formation of electrical impulses

Average Expenditure of Energy in the Formation of Electrical Impulses

The values for these curves are obtained from the product of the square of the percentage average potential per half-hour and the percentage average rate for the same half hour (divided by 10,000 to bring it to a percentage) The curves of these values plotted against time in culture (Fig 5) for both studies show initially low values—possibly the effect of tissue 'breakdown' products from explantation Other factors which may contribute in a lesser degree are the drop in temperature from the egg (37 5° C) to that in the incubator (37° C), also the cooling of the eggs and the embryo during the actual process of culturing The expenditure of energy then climbs to its highest level largely due to the high rate of contraction at this time in culture The initial double peak seen best in study 2 is probably the result of the innate cyclic activity of the heart tissue The subsequent drop in energy expenditure is *not* due to the loss of electrical function in culture since the energy curve flattens out before the electrical persistence curve does A comparison of the average electrical energy expenditure curves with those for potential per contraction and contractile rate suggests that each explant

may have available to it a certain amount of available energy per unit of time for the formation of electrical discharges and that this amount of energy becomes less with the passage of time The limitation on the amount of available energy may be the result of a limited supply of essential metabolites (co-factors etc) The eventual lossening of the amount of available energy may be due to a progressive utilisation of a limited supply of these metabolites Initially the hearts seem to use the energy in the formation of a large number of relatively low potential contractions and subsequently in the formation of a smaller number of relatively high potential beats The intervals between the peaks of cycles in the energy expenditure curves are 20 hours for both studies

The Cyclic Activity of Cardiac Tissue in Culture

The cyclic activity is more evident in study 2 probably because all of the explants were made about the same time of day whereas in study 1 half of them were made about 10 a m and the other half about 3 p m The greater frequency of observation in study 2 delineated the cycles more accurately However the intervals between the peaks of the cycles were judged visually and are only approximate The average duration of all peaks judged visually was about 5 hours The average interval between all cycles observed in both studies was about 20 hours The approximate nature of the measurements makes it possible that the cycle is in fact a 24 hour one which would bring it more into line with the usual biological rhythms When more data is available more precise mathematical methods will be used to determine the exact duration of these cycles

Period of Greatest Stability of Heart Tissue in Cultures

The data in study 1 indicated that the period of greatest stability of electrical activity of heart tissue in culture was between 100 and 133½ hours after explantation The additional data now available including that on the persistence of electrical function in culture (*Cunningham Fanell & Ryland ler* 1961) suggests that the period of gre test st [illegible]
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SUMMARY

The normal electrical behaviour of 107 whole heart explants from 7-day chick embryos kept for 6 days in tissue culture was studied Each heart was kept in its own culture chamber [illegible] d with a flow of
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impulses from the hearts. The data concerning potential rate, potential per beat and energy expenditure was collected and interpreted. From the graphs obtained it is suggested that cardiac tissue in culture has an inherent property of daily cyclic variation. The period of greatest stability and thus the period most suitable for experimental manipulations seems to be between 75–91 hours or after 125 hours in culture.

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HISTOCHEMISTRY OF THREE DEHYDROGENASE SYSTEMS IN CANCEROUS AND NON-CANCEROUS HUMAN STOMACHS WITH SPECIAL REFERENCE TO INTESTINAL METAPLASIA

By

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Received 6 III 61

The enzyme histochemistry of various human tumors has been intensely studied recently (*Monis et al* 1959 a, b and 1960, *Glenner et al* 1959, *Hannibal et al* 1960) Of the tumors of the human gastrointestinal tract those occuring in the large intestine have been carefully investigated by *Wattenberg* (1959) While the study presented in this paper was in progress a most interesting study on the histochemistry of some enzymes in the normal and metaplastic human stomachs appeared (*Planteydt & Willighagen* 1960)

The localization of diphosphopyridine nucleotide (DPN) diaphorase and lactic and succinic dehydrogenases in the normal human gastric mucosa was described in our previous paper (*Niemi et al* 1960) We have since applied the same histochemical reactions on specimens obtained from cases of gastric carcinoma and peptic ulcer, and the results of these studies will be presented here The distribution of the enzyme activities in the intestinal and pseudopyloric metaplasia has been particularly considered

MATERIAL AND METHODS

The material consist of 20 gastrectomy specimens, in seven cases there was a carcinoma in the pyloric part of the stomach in three the [illegible]

[illegible] ars, seven of [illegible] younger than [illegible] rately in the [illegible] nto three to [illegible] gastric lesion as well as [illegible] Scarpelli & Pearse [illegible]

(2) Lactate dehydrogenase (LD) (*Scarpelli Hess & Pearse* 1958)
(3) Succinate dehydrogenase (SD) (*Nachlas & Seligman* 1957)
One serial section of each tissue block was stained with hematoxylin and eosin
In a few occasions pieces of tissue were fixed overnight in formol calcium where after DPND and LD were demonstrated on frozen sections

RESULTS

Intestinal metaplasia was present in each of the gastrectomy specimens obtained from patients with gastric carcinoma In seven neoplastic cases tubules resembling morphologically pyloric glands and containing no zymogenic cells were found in the mucosa of the body of the stomach, these tubules were therefore taken according to *Schindler* (1947) as examples of pseudopyloric metaplasia The corresponding incidence of the two types of metaplasia among the nine ulcer patients was six and four, respectively

Malignant cells with a distinct striated border were seen *in one case* and in another one the gradual transition of metaplastic epithelium into neoplastic could be clearly seen

In most of the specimens some of the sections stained with H & E revealed a fairly normal looking pyloric mucosa, the corresponding serial sections were therefore taken as normal controls when the patterns of enzyme activities were evaluated

TABLE 1
Dehydrogenase Activity of Carcinomatous Cells as Compared with that of the other Components of Gastric Mucosa

	No of cases	Lactic dehydrogenase				Succinic dehydrogenase				DPNH diaphorase			
			+	++	+++		+	++	+++	−	+	++	+++
Carcinomatous cells	*10*	−	2	6	1	3	5	1		−	8	2	−
Parietal cells	10	−			9	−	−	−	9		−	2	8
Surface epithelial cells of the body of the stomach	10	4	2	2	1	8	1			5	5		
Intestinal metaplasia	*10*		1	4	4		1	2	4		3	5	2

Dehydrogenase Activity in the Malignant Tissues

The qualitative and quantitative distribution of the three oxidative enzymes studied in carcinomatous tissue is given in Table 1 The visual quantitation was based on the degree of staining of parietal cells (Figs 1 and 2), which were present in every specimen and gave always a strong and consistent staining (*Niemi et al* 1960)

It can be seen from Table 1 that the activities of LD, SD and DPND were generally weaker in neoplastic cells than in normal parietal or even metaplastic cells (Figs 1 4) However, the enzyme activity of the cancerous tissue was *found to be higher than that of the* surface epithelial cells outside the neoplastic area Lactate dehydrogenase activity



F gs 1 7

F g 1 Succ n c d hy lr genase act vity in the stomach of a cancer patient A few tubulus with parietal cells exhibit ng strong enzyme act vity can be seen in upper part f the p tur The middle and l wer part f the picture show the neglig ble a t ity f the carc n ma ti sue × 38 *F g 2* SD activity is strong in the parietal cell whereas the surr un ing carcin mat us tis ue do n t stain at all × 38 —

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(Fig. 4) was usually relatively higher than the activity of succinate dehydrogenase. Moreover, in three of the cancer specimens SD activity was absent or extremely weak. The staining of the malignant cells at the borderland of the tumor did not essentially differ from that of the cancerous cells elsewhere.

No constant differences could be observed in the staining properties between the highly anaplastic and the more differentiated forms of gastric carcinoma. However, in two of the less anaplastic cases the activity of all the three oxidative enzymes seemed to exceed that of any of the anaplastic tumors.

The case of malignant lymphoblastoma included in the series showed a quite different picture: no enzyme activity at all was demonstrable in the malignant lymphatic cells, whereas the normal mucosa surrounding the tumor stained in the usual way.

TABLE 2
Dehydrogenase Activity of Intestinal Metaplasia

Intestinal metaplasia in	No. of cases	Lactic dehydrogenase −	+	++	+++	Succinic dehydrogenase −	+	++	+++	DPNH diaphorase −	+	++	+++
Gastric carcinoma	10	–	1	4	4	–	3	2	4	–	3	5	2
Peptic ulcer	6	–	1	2	3	–	1	5	–	–	1	5	–
Whole material	16	–	[illegible]	6	7	–	4	7	4	–	4	10	2

Dehydrogenase Activity in Metaplasia

Intestinal metaplasia. In most of the sections studied there was present intestinal metaplasia. The distribution of the oxidative enzymes in metaplastic tissues is summarized in Table 2. In all the specimens the intestinal tubules showed activities of all the three enzymes, LD activity being usually more pronounced than the two others. Generally the metaplastic epithelium exhibited slightly stronger activities than the corresponding normal intestinal epithelium (Figs. 5 and 6). Some differences were, however, encountered in the distribution of the enzymes in the intestinal tubules. LD exhibited usually most intense activity in the superficial part of the tubule, the middle portion being less intensely stained (Figs. 5, 6, 10 and 11). In some tubules the bottom and the superficial parts were equally stained (Fig. 11). DPND showed usually almost the same pattern of distribution as LD. On the other hand SD activity was found to be highest in the bottom of the metaplastic tubules, but in the normal mucosa of the jejunum the surface epithelium was the site of maximal staining.

No difference could be noticed between intestinal metaplasia of cancerous and non-cancerous stomachs. The enzymatic activity was localized to the striated border cells only, the goblet cells giving negative staining reaction. Paneth cells could not be differentiated.



Figs 8 11

F g 8 I seud p l ric metaplasia in a st mach with pept c ulcer The meta lastic tul ules are surrounde l w th normal gastric tubules LD reaction × 78 —*Fig 9* LD activity in n rmal p l ric muc sa × 48 —*Fig 10* A l w p wer view f a secti n fr m a ne pla tic st mach LD activity is p r minent in the metaplastic tubules The can er ti sue in the m dle f the picture g ves negative stain ng × 38 —*F g 11* Di stributi n of SD activity in two meta last c indestinal tul ules × 31

Pseudopyloric metaplasia This form of metaplasia was present in seven of the specimens from cancer patients and in four from the patients with peptic ulcer Metaplastic tissue was found, however, enzymatically inactive in most cases (Fig 8) Sometimes a weak staining of a few tubules was observed, but its distribution differed considerably from that of a normal mucosa of pylorus In a metaplastic tubule the enzyme activities, if present, were equal at all levels of the tubule whereas in a normal pyloric tubule (Fig 9) SD activity was clearly concentrated to the middle portion of the tubule, DPND and LD being most active in the bottom of the tubules

Other elements of the gastric mucosa The superficial epithelium was generally enzymatically inactive in every part of the gastric mucosa However, in connection with gastritis and a pronounced inflamatory reaction a moderate LD activity could be seen even in the superficial epithelial cells of the body mucosa

DISCUSSION

The possible differences in oxidative metabolism between neoplastic and normal tissue have been much discussed lately (*Meister* 1950, *Greenstein* 1954, *Weinhouse* 1955) Many of the earlier works cited have been done on tissue homogenates, but even histochemical approach has been widely used (*Goddard & Seligman* 1953, *Monis et al* 1959, *Hannibal et al* 1960) The conclusion is drawn in most of these studies that there are no striking differences in the oxidative metabolism of the two types of tissues

In our present work the gastric carcinoma cells were found to exhibit less activity of three dehydrogenases than the normal parietal cells This finding is in agreement with the general observation of *Monis et al* (1959) that the human neoplastic cells show slight but enormously variable amounts of these enzymes In our gastric specimens we could not, however, observe any considerable variation between neoplastic cells of a given carcinoma The borderland of a given tumor did not show any differences in its enzyme activity as compared with the more central tissue of the same tumor No correlation was even seen between dehydrogenase activity and the degree of cell differentiation

The observations on the enzyme distributions in metaplastic gastric epithelium are of interest since metaplastic changes have been considered as precancerous in gastric mucosa (*e g Morson* 1955) The findings made in the present study show clearly that the localization of the three oxidative enzymes in the intestinal metaplasia corresponds well with that in the normal small bowel There were only some differences in the distribution of SD, which might not necessarily indicate significant variations We could therefore conclude that the metaplastic epithelium of the stomach resembles both morphologically and enzymatically the epithelium of the gut There were nothing common between

the enzyme distributions in the metaplastic and normal gastric mucosa Exactly the same conclusion is reached by *Planteydt & Willighagen* in their recent contribution (1960)

From the point of view of gastric carcinogenesis it is of value to see whether the intestinal metaplasia present in cancerous and non cancerous stomachs exhibit any differences As has been shown in our Table 2, no significant differences could be demonstrated in this respect In both, the obviously functionally active intestinal epithelium might have an influence on the pathogenesis and the clinical course of the diseases

SUMMARY

The localization of three oxydative enzymes (diphosphopyridine nucleotide diaphorase and succinic and lactic dehydrogenases) has been studied in gastrectomy specimens from patients with gastric carcinoma and peptic ulcer

The neoplastic tissue was found to have less activity of all the enzymes than the normal parietal cells The carcinoma cells showed, however, moderate lactic dehydrogenase activity, some diaphorase activity, but negligible succinic dehydrogenase activity

The distribution and localization of the enzymes in intestinal metaplasia was equal with that in the normal epithelium of the bowel No differences were noticed in the enzyme pattern of metaplasias in cancerous and non cancerous stomachs

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THOROTRAST TUMOURS

A Review of the Literature and Report of two Cases

By

SVEN DAHLGREN

Received 24 iii 61

Although thorotrast was used as a contrast medium to a fairly wide extent in Scandinavia as late as the 1940s, there has been no report from these countries of malignant tumours ascribed to this technique. Since the latency period for malignant thorotrast tumours ranges from 10 to 35 years it would seem likely that in the next few years a large number of such cases will be encountered. In recent years a number of such cases have been reported from other countries.

In connection with the description of two new cases, one of a fibrosarcoma at the site of injection and the other cancer of the bile ducts, a detailed study of the literature in the field has been made.

Thorotrast is a colloidal solution of thorium dioxide (ThO_2), containing about 25 per cent of the dioxide with a particle size of between 3 and 10 mμ, 25 per cent of partly inverted carbohydrates and 50 per cent water, with small amounts of preservatives. Thorium has a half life period of 1.4×10^{10} years, and 20 ml of thorotrast are equivalent in radioactive effect to about one μg radium.

The radiation emitted by thorium consists of 90 per cent alpha, 9 per cent beta and one per cent gamma rays (*Johansen* 1954).

Survey of the Literature

Thorotrast deposited in the tissues can be demonstrated histologically. In sections stained by van Gieson's method particles of thorotra[illegible]

[illegible] specific for thorotrast. The particles can, however, be recognized by autoradiography and determination of the radiation spectrum (*Roberts & Carlson* 1956).

Soon after its introduction in 1928 this medium won favour in many different spheres of radiology, including myelogr[illegible]
gr[illegible]
ang[illegible]
but [illegible] most commonly used, especially in Sweden

The possibility of radioactive injury from thorotrast, in the first place cancer, and then damage to the vessels, was *recognized shortly after* its introduction, but in spite of persistent warnings, thorotrast gained in popularity owing to its advantages over media used hitherto, chiefly the absence of vascular *reactions and allergic complications Moreover*, it is very homogeneous

In comparatively few instances have clinical complications been reported (*Thomas et al* 1951, *Backer* 1957, *Johansen* 1960), but *indurations* have been found to form readily at the site of injection Sporadic cases of anaemia and hepatic disorders have been described, and follow-up examinations *have revealed histologic changes in the liver, spleen*, bone marrow, skeleton and other organs, chiefly in the form of fibrosis, necrosis and granulomas (*Rotter* 1951) *Lindgren* (1938) described cerebral damage *after arteriography with the medium In 21 cases out* of 35 there was histologic evidence of thorium in the cerebral vessels and in 6 of these there were small haemorrhagic areas and softenings In *several countries, including Sweden, the use of thorotrast* was discontinued in the 1940s, but in other countries this was still being recommended as one of the foremost media far into the 1950s (in, for instance, *angiography*)

Early damage with this substance is slight Study of long-term effects, especially growth of malignant tumours, requires protracted observation of the patient, *and for this reason they have in the past* not received due attention The increasing use of radioactive materials in recent years, however, has attracted focused interest on the subject of thorotrast tumours

As early as 1935 46 it was demonstrated on laboratory animals that thorotrast injected intravasally, interperitoneally and subcutaneously could give rise to tumours (*Selbie* 1936, *Foulds* 1939, *Guimaraes et al* 1955 and *Johansen* 1954) The first case of thorotrast tumour in man (endothelial-cell sarcoma of liver) was discovered in 1946 by *MacMahon et al* (1947) and since then some 60 cases of such tumours have been described

These may be divided into three groups according to their mode of origin

(A) Tumours appearing at the site of injection (Table 1)

(B) Tumours due directly to thorotrast deposited in the hollow organs, etc (Table 2)

(C) Tumours due to thorotrast injected systemically and deposited *in the reticuloendothelial system* (Table 3)

For the tumour to be ascribed to thorotrast deposited in the tissues the following conditions should be satisfied (I) Thorotrast particles should be found in the immediate vicinity of the tumour (II) *The* latency period should be sufficiently long and (III) the radiation dose

Tumours at Site of Injection

Author		Tumour	Purpose of Injection	Dose cc	Lat per year	Sex	Pt Age* year
Plenge et al	1954	Fibroblastsarcoma	Cerebral angiography	20	6	F	54

* Age at diagnosis or death

TABLE 2
Tumours after Deposition of Thorotrast in Performed Cavities

Author		Tumour	Purpose of Injection	Dose cc	Lat per year	Sex	Pt Age* year
Zollinger	1949	Spindel cell sarc kidney	Pyelography	30	16	M	64
Austoni	1950	Carc breast	Mammography	10	12	M	47
Ruf & Philipp	1950	Carc eyelid	Dacryocystography	–	35	M	51
Hefer	1952	Carc maxillary sinus	Maxillary visual	–	10	F	64
Vogtlin & Minder	1952	Bronchogenic carc	Bronchography	30	18	M	47
Gros et al	1955	Carc maxillary sinus	Maxillary visual	–	15	M	36
Scheibe	1955	Malign perit thorotrastoma	Intraperitoneal	24	17	F	60
Boemke	1956	Carc kidney	Pyelography	–	–	M	75
Budin & Gershon Cohen	1956	Carc breast	Mammography	–	17	M	43
Federlin & Scior	1957	Carc ovary	Salpingography	–	23	F	45
Jacob & Schostek	1957	Carc kidney	Pyelography	–	25	F	49
Roth	1957	Bronchogenic carc	Bronchography		13	M	38
Nielsen & Knacht	1958	Mixed tumour kidney		–	–	F	66
Verhaak	1958	Adenocarc kidney	Pyelography		21	F	43
Verhaak	1958	Carc in situ kidney	Pyelography	–	23	F	44
Gelzer & Scheidegger	1959	Carc seminal vesicle	Urethrovesiculography		14	M	68
Aiken et al	1960	Carc kidney	Pyelography	–	23	M	56
Cattell & Kahn	1960	Peritonealcarc	Salpingography		21	F	48
Gros et al	1960	Carc maxillary sinus	Maxillary visual	–	14	F	49
Kligerman et al	1960	Carc maxillary sinus	Maxillary visual	–	18	F	32
Kligerman et al	1960	Carc maxillary sinus	Maxillary visual		21	M	39
Kligerman et al	1960	Carc maxillary sinus	Maxillary visual	–	10	M	70
Kligerman et al	1960	Carc maxillary sinus	Maxillary visual	–	15	F	70
Kruckemeyer et al	1960	Carc kidney	Pyelography	–	23	F	45

* Age at diagnosis or death

should be sufficiently high (The measurement of the radiations should preferably be performed on the organ concerned)

In the following compilation only malignant tumours have been included

In group A there is only one case of malignant tumour There are, however, cases of benign fibrous tumours belonging to this category (*Rotter* 1951) These appeared in direct connection with the thorotrast particles deposited extravasally at injection

Group B is fairly heterogeneous since thorotrast was used in so many different types of radiologic examination A common feature of all these cases, however, is that the medium was deposited in cysts, fistulas, performed cavities, etc, and after the examination all the contrast medium injected had not been removed

It should be noted here that the cases atrributed to *Ruf & Philipps* were also reported by *Rudolphi* in 1951 Similarly, *Budin & Gershon-Cohen*'s patient was published by *Brody & Cullen* (1957), *Scheibe*'s case by *Klemm* (1955) and *Federlin & Scior*'s by *Schwenzer & Federlin* (1957)

In the group C cases, in which malignant tumours were due to deposit of thorotrast in the reticuloendothelial system after systemic injection, the contrast medium was used chiefly in cerebral angiography and hepatolienography The tumours were practically all localized in the liver but a few cases are described where they appeared in other organs in contact with the thorotrast foci in the liver, spleen or lymph nodes (Group 3 B) Cases of leukaemia ascribed to thorotrast deposits are borderline ones of this group but are not included in this review (*Wohlwill* 1942, *Spier et al* 1947, *Grebe* 1954, *Netousek* 1957)

Thorotrast is taken up in the reticuloendothelial system and deposited chiefly in the liver, spleen and bone marrow in the ratio 7 5 1 (*Rossle* 1949) A very small amount was also deposited in the lungs and the adrenals The amount excreted is insignificant The particles of thorotrast are deposited as large aggregates, which gradually increase in size

In the spleen the changes do not take the form of tumour growth but of progressive destruction with fibrosis of the parenchyma The spleen is usually reduced in size and has a firm consistency (*Looney* 1955 *Looney & Colodzin* 1956) Vascular changes in the spleen have also been described (*Levy* 1960)

Twenty six cases of different forms of malignant tumours originating in the liver or bile ducts after use of thorotrast have been reported

An increasing number of cases of liver tumours following application of thorotrast have been reported in recent years, and more can be expected in the immediate future

If *Silva Horta*'s first case (1953) is omitted on the grounds of the short latency period of 3 years 2 months, the average period for the 28 cases published is about 18 years In 1951 *Thomas et al* described a case of liver tumour that had appeared 9 years after hepatolienography

TABLE 3 A

After Systemic Injection. Malignant Liver and Bile Duct Tumours

Author		Tumour	Purpose of Injection	Dose cc	Lat per year	Sex	Pt. Age* year
MacMahon et al	1947	Endothelial-cell sarc	Hepatolienography	75	12	F	70
Fudin	1953	Endothelial cell sarc	Hepatolienography	75	14	F	63
Silva Horta	1953	Endothelial cell sarc	Cerebral angiography	20	3 2	F	–
Heltman	1954	Carc common duct	Hepatolienography		20	M	39
Matthes	1954	Bile duct carc	Hepatolienography	80	21	F	54
Frühling	1955	Endothelial cell sarc	Arteriography	–	12	M	49
Teslik & Nordin	1955	Hemangioendothelioma	Cerebral angiography	–	14	M	54
Carli et al	1956	Hemangioreticuloma	Arteriography	–	24	M	58
Grossiord et al	1956	Adenocarcinoma	Arteriography	80	21	M	54
Matthes	1956	Hepatocellular carc	Hepatolienography	80	23	F	53
Matthes	1956	Cholangiocellular carc	Hepatolienography	70	23	F	52
Roberts & Carlson	*1956*	*Hepatic duct carc*	Arteriography	–	17	F	45
Silva *Horta*	1956	Endothelial cell sarc	Arteriography	–	22	M	46
Batzenschlager & Wilhelm	1957	*Primary carc*	Arteriography	–	12	M	48
Federlin & Selor	1957	Primary carc	Cerebral angiography	–	13	M	47
Okinaka et al	1957	*Carc common duct*	–	–	11	F	40
Grampa & Degna	1958	Hemangioendothelioma	Hepatolienography	75	23	M	61
Hieronymi	1958	Hemangioendothelioma	Arteriography	–	15	M	49
Hieronymi	1958	Bile-duct carc	Arteriography	–	16	M	61
Morgan et al	1958	Primary carc	Hepatolienography	–	24	F	48
Gardner & Ogilvie	1959	Carc left hepatic duct	Cerebral angiography	–	22	F	47
Rosenbaum	1959	Hemangioendothelioma	Arteriography	80	13	M	49
Wyrthemann	1959	Carc liver and lung	Arteriography	–	20	M	57
Haserga et al	1960	Bile duct carc	Livercyst visual	–	14	M	17
Gros et al	1960	Endothelio angiosarcoma	Arteriography		12	M	49
Kohoutek & Novak	1960	Cholangiogenous carc	Hepatolienography	75	24	M	61
Stemmermann	*1960*	*Adenocarcinom*	Arteriography	–	20	M	58
Stemmermann	1960	Adenocarcinom	Arteriography	–	20	F	56

* Age at diagnosis or death

TABLE 3 B

After Systemic Injection Malignant Tumours other Sites than Liver and Bile Ducts

Author		Tumour	Purpose of injection	Dose cc	Int per year	Sex	Age* year
Budin & Gershon Cohen	1956	Carc colon	Hepatolienography			F	65
Hackenthal	1956	Bronchogenic carc	Arteriography		16	M	75
Wuketich & Mark	1957	Carc pancr and kidney	Arteriography		11	M	70
Nielsen & Kracht	1958	Adenocarc lung	Hepatolienography	–	24	M	43
Gardner & Ogilvie	1959	Giant follic lymph spleen	Cerebral angiography	15	15	F	65
Friedrich	1960	Carc kidney	Cerebral angiography	–	20	F	55

* Age at diagnosis or death



Fig 1
Case 1 Neurofibrosarcomatous tumour with small deposits of thorotrast (van Gieson) × 167

with thorotrast This case has not been included in the above compilation because it seems not to have been sufficiently thoroughly described Doubt attaches to *Stemmermann*'s second case (1960) as this patient had an associated cystadenocarcinoma of the ovary

Many compilations of thorotrast tumours include a case described by *Abrahamson et al* a 63 year old woman who 16 years after hepatolienography with thorotrast (7 ml) died in bilateral alveolar lung carcinoma Since no thorotrast was found in the lungs this case cannot be ascribed to the thorotrast and has therefore not been included in the present review Doubt attaches also to *Budin*'s (1956) case especially in view of the fairly high spontaneous incidence of cancer of the colon The mean latency time in this heterogeneous group (III B) was about 18 years

REPORT OF TWO CASES

Case 1

A man aged 41 Head injury in 1932 epileptic fits began in 1933 He was admitted to a hospital in 1941 for neurological examination

Encephalogrammes suggested a brain tumour situated on the left side of the convexity Arteriography with thorotrast via the left common carotid artery (amount of medium is not reported) showed arteriovenous aneurysm confirmed by histological examination

In 1951 the patient discovered a nodule in the soft tissues on the left side of the neck Test excision showed neurofibroma of a benign type and an attempt was made to remove the tumour Histologic examination showed neurofibroma consisting of tumour tissue with regions poor in cells and rich in collagen alternating

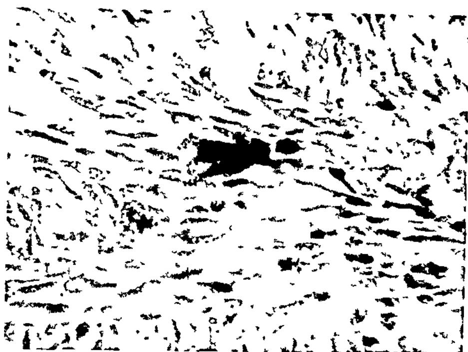


Fig 2
Case 1 Deposits of thorotrast situated in macrophages and surrounded by neurofibrosarcomatous tumour (van Gieson) × 448

with regions rich in cells and poor in collagen There was no evident palisade disposition Cellular polymorphism was fairly slight There were sporadic mitoses Small deposits of thorotrast were observed

Radiologic examination In 1960 tomography of the soft tissues of the neck suggested thorotrastoma There were no definite metastases The spleen was greatly enlarged and the parenchyma contained miliary densifications which could have been due to thorotrast Similar densifications were observed around the porta hepatis which may have been due to thorotrast accumulations in the lymphatic nodes

15th February 1960 Sternal puncture showed numerous particles in the marrow with a reduced fat content and increased cellular activity The bone marrow was markedly hyperplastic The sections showed a dense cell agglomeration of cells that contained thorotrast

18th February and 12th December 1960 Further attempts were made to remove the tumour since it had increased in size and was considered to be locally malignant

Histologic examination (performed at the Institute of Radiopathology Stockholm) showed a partially encapsulated tumour resembling a neurofibroma and parts of which diffusely merged with connective tissue and fat tissue suggestive of an infiltrative tendency In places there were regions fairly density in cells with some polymorphous nuclei and mitosis The cells in both density and disposition suggested a highly differentiated fibrosarcoma (neurofibrosarcoma) In and around the tumour tissue there were numerous deposits of thorotrast some of them situated in macrophages (Figs 1 2)

To summarize, the patient was a 41 year-old man on whom at the age of 21 cerebral angiography had been performed with thorotrast since 1959 he had had recurrent neurofibrosarcomatous tumours in the neck at the site of injection of the medium

Case 3

A man aged 64 who in 1938 underwent cerebral angiography for examination of headache and epileptic fits from which he had been suffering for some 8 years. Bilateral arteriography via the internal carotid artery performed with 4 × 8.5 ml th rotrast disclosed an aneurysmal widening of part of the uppermost branch of the medial cerebral artery on the right side. In 1958 the patient was admitted to hospital for left pleural pneumonia and cardiosclerosis and arthrosis deformans. Radiology then showed a radiopaque thorotrast spleen. In 1959 the man was re-admitted to hospital with diffuse aching throughout the body and high temperature. Anaemia was noted with Hb 55–60 per cent, erythrocytes 3.5 million, white cells 15 600–20 000. The liver was enlarged. Needle biopsy from the liver showed cells that were

ated rapidly and he died in the autumn of

External examination. Left leg oedematous and bluish in colour. Otherwise normal. No jaundice.

Circulatory system. The he

no malformations. Coronary

A thrombus attached to the

Respiratory organs. The

tumour. Lungs heavier than ... On the basal pleural

... the palm of the hand.

The thyroid gland was fairly much enlarged and weighed 160 g. The surface of section was homogeneous, smooth and a light greyish brown colour. No evidence of malignancy.

... adherent to the spleen.

The stomach and intestinal tract showed no definite pathologic changes. No evidence of tumour.

The liver weighed 1850 g and extended down below the right ...

There were no

Femur. The bone marrow was active with no metastases.

Cran...

from ...

cerebra...

near t...

Micr... examination. Sections from the large tumour infiltrate in the region of the hilus showed pictures of a ...

tumour was composed of larg...

parts which contained fairly

of the tumour showed a ...

ular there were large areas ...

The tumour involved both blood and lymph vessels and nerve sheaths. Areas of fibrous tissue contained large quantities of thorotrast particles, some of them

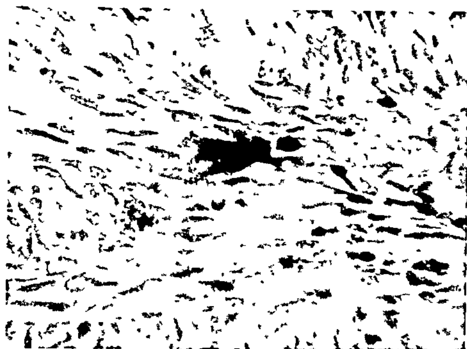


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C se 2

A man agc l 64 wh in 1938 underwent cerebral angiographs f r examinati n of headache an l epilept c fits from which he had been suffering f r s me 8 years B lateral arteriographs via the internal car t d artery performed with 4 × 8 5 ml th r trast d closed an aneurysmal widen ng of part of the upperm st branch f the med al cerel ral artery n the r ght side In 1958 the pat ent was admitted to h spital f r left pleural pneumon a and card scleros s and arthr sis deformans Ra li logy then sh v e l a rad opaque thor trast spleen In 1959 the man was re admitted to h sp tal with d ffuse aching throughout the l ody and high temperature Anaemia was n ted w th Hb 55 60 per cent erythrocytes 3 5 million wh te cells 15 600 20 000 The liver was enlarged Nee lle b psy from the l ver sh wed cells that were su pected to be mal gnant

The c nd t on of the patient leteriorated rapidly and he died in the autumn of 19 9 (s th a p cture of cachex a)

Autopsy at the Karol nska sjukhuset

External exam nati n Left leg oe lematous an l l lu sh in c l ur Otherw e n r mal N jaun l ce

C rculatory system The heart was n rmal in size and c nf gurati n There v ere n malf rmations C ronary vessels the a rta and pulmonary arteries were n rmal A thr mbus attached to the wall of the left fem ral vein ccluded the l men

Re p ratory organs The resp rat ry tract was unobstructed N   ev dence of

c a   l t t and

The thyr id gland was fa rly much enlarged an l weighe l 160 g The surface of ection was hom geneous sm oth and a l ght greyish br wn c lour No evi lence f malignancy

at   a   a   adherent to the spleen

The stomach and intest nal tract sh wed n   lefinite path l gic changes N evi len e of tumour

The liver we ghed 1850 g and extende l d wn l cl w the right b r ler of the ribs On the surface f the l ver were several round greyish wh te foci typ cal f meta stases The urface of secti n showed numerou metastatic f ci up to 3 cm in d ameter s me f wh ch c nta ned small quantit es of a greyish opalescent muc is At the hilus there was a l ffusely lemarcated f rm greyish white tumour al ut cm in d am ter and gr wing d wn t ward the h ad f h

There were n

Femur The b ne marrow w   a

Cranium and men nges

fr m a 1 5 × 1 5 cm r und

c rel ral arter   2 cm fr m

near the f c s sh wc l sn

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cu ar th re were large areas f necr sis   au l car these reg ns in parti

The t im r ins lve l b th f l   d and lymph ves els and nerve sheaths Areas of f l r us ti s e c ntained large quantit es f th r trast part cles some of them

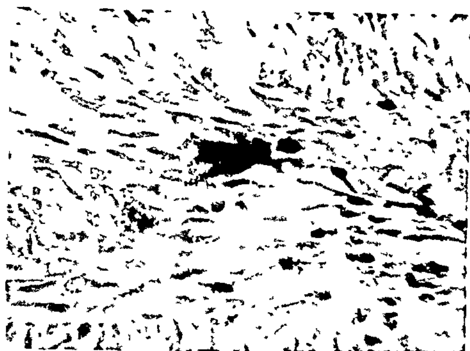


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Case 1 Deposits of thorotrast situated in macrophages and surrounded by neurofibrosarcomatous tumour (van Gieson) × 448

with regions rich in cells and poor in collagen There was no evident palisade disposition Cellular polymorphism was fairly slight There were sporadic mitoses Small deposits of thorotrast were observed

Radiologic examination In 1960 tomography of the soft tissues of the neck suggested thorotrastoma There were no definite metastases The spleen was greatly enlarged and the parenchyma contained miliary densifications which could have been due to thorotrast Similar densifications were observed around the porta hepatis which may have been due to thorotrast accumulations in the lymphatic nodes

15th February 1960 Sternal puncture showed numerous particles in the marrow with a reduced fat content and increased cellular activity The bone marrow was markedly hyperplastic The sections showed a dense cell agglomeration of cells that contained thorotrast

18th February and 12th December 1960 Further attempts were made to remove the tumour since it had increased in size and was considered to be locally malignant

Histologic examination (performed at the Institute of Radiopathology Stockholm) showed a partially encapsulated tumour resembling a neurofibroma and parts of which diffusely merged with connective tissue and fat tissue suggestive of an infiltrative tendency In places there were regions fairly density in cells with some polymorphous nuclei and mitosis The cells in both density and disposition suggested a highly differentiated fibrosarcoma (neurofibrosarcoma) In and around the tumour tissue there were numerous deposits of thorotrast some of them situated in macrophages (Figs 1 2)

To summarize the patient was a 41 year old man on whom at the age of 21 cerebral angiography had been performed with thorotrast since 1959 he had had recurrent neurofibrosarcomatous tumours in the neck at the site of injection of the medium

deposited in macrophages The metastases in the liver contained numerous vegetations of the same type of primary foci Different degrees of differentiation were also found in the metastases Owing to fairly extensive postmortem changes it was difficult to asses the uninvolved liver parenchyma but there were fairly many thorotrast deposits especially round the centrilobular vessels There were signs of mild stasis and fatty degeneration No cirrhosis (Figs 3 4)

Pancreas The tumour tissue at the hilus of the liver grew down towards the pancreas which however appeared to be intact and its structure normal No signs of thorotrast deposits

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spleen There was an appreciable thickening of the capsule and the deepest parts contained numerous deposits of thorotrast At one place in the capsule there was a small focus with growth of the same cancer described in the liver

Bone marrow from vertebrae Largely normal structure No metastases The sections showed however fairly numerous thorotrast particles some of them extracellular and some in large macrophages

Bone marrow from the femur The sections showed signs of increased haematopoetic activity No tumour No thorotrast deposits possibly due to the fact that at the time of the injection of thorotrast the femur marrow was probably *not haematopoetically* active but consisted of fat When the bone marrow originally active on *the* occasion of the injection was subsequently damaged by the thorotrast radiation the femur marrow was activated Since however no thorotrast was found in the *blood* vessels at this later occasion none could have been deposited

Lungs Signs of stasis and fairly advanced oedema In places small bronchopneumonical *infiltrates* No evidence of tumour No definite signs of thorotrast deposits Round the vessels and *especially subpleurally* there were moderate anthracotic deposits The subpleural parenchyma of the lung showed increased fibrosis The small reddish brown *formations on the* visceral pleural surfaces consisted of gran...

... *evidence of* metastases there was growth of tumour similar to that *found in the liver* The tumour tissue in the metastases however was strikingly necrotic and in places imbibed with blood Small foci of thorotrast particles *were seen in the* lymph nodes

Kidneys Moderate arterio and *arteriolosclerosis* There were no thorotrast deposits

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In brief, a man of 64 years, who 21 years previously had had injection of thorotrast in connection with cerebral angiography At autopsy thorotrast deposits were found in the liver, spleen, bone marrow and lymph nodes In connection with the large foci of thorotrast in the h... of the ...
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in the ... *patient died* in secondary heart failure

DISCUSSION

In both cases the thorotrast tumours were probably elicited by radiation from thorium particles deposited *in* the tissues As regards the latter case, where there was cancer of the bile duct, importance attaches

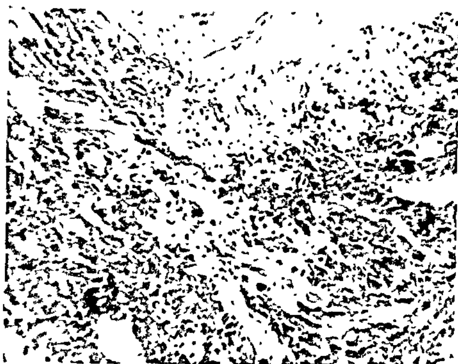


Fig 3
Case 2 Adenocarcinoma in the liver containing large deposits of thorotrast Dense masses in the lower portion represent the thorotrast (van Gieson) × 167

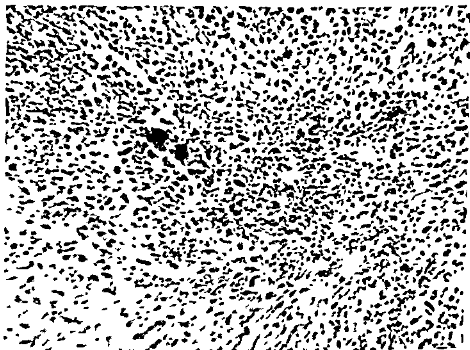


Fig 4
Case 2 Metastas in the liver showing intermixture of carcinoma and thorotrast (van Gieson) × 167

deposited in macrophages. The metastases in the liver contained numerous vegetations of the sam type of primary foci. Different degrees of differentiation were also found in the metastases. Owing to fairly extensive postmortem changes it was difficult to asses the uninvolved liver parenchyma but there were fairly many thorotrast deposits especially round the centrilobular vessels. There were signs of mild stasis and fatty degeneration. No cirrhosis (Figs. 3, 4).

Pancreas. The tumour tissue at the hilus of the liver grew down towards the pancreas which, however, appeared to be intact and its structure normal. No signs of thorotrast deposits.

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contained numerous deposits of thorotrast. At one place in the capsule there was a small focus with growth of the same cancer described in the liver.

Bone marrow from vertebrae. Largely normal structure. No metastases. The sections showed however fairly numerous thorotrast particles, some of them extracellular and some in large macrophages.

Bone marrow from the femur. The sections showed signs of increased haematopoietic activity. No tumour. No thorotrast deposits, possibly due to the fact that at the time of the injection of thorotrast the femur marrow was probably not haematopoietically active but consisted of fat. When the bone marrow, originally active on the occasion of the injection, was subsequently damaged by the thorotrast radiation the femur marrow was activated. Since, however, no thorotrast was found in the blood vessels at this later occasion none could have been deposited.

Lungs. Signs of stasis and fairly advanced oedema. In places small bronchopneumonial infiltrates. No evidence of tumour. No definite signs of thorotrast deposits. Round the vessels and especially subpleurally there were moderate an

...also showing macroscopically evidence of metastases there was growth of tumour similar to that found in the liver. The tumour tissue in the metastases, however, was strikingly necrotic and in places imbibed with blood. Small foci of thorotrast particles were seen in the lymph nodes.

Kidneys. Moderate arterio and arteriolosclerosis. There were no thorotrast deposits.

Thyroid gland. Acini enlarged and rich in collagen, though with no evidence of increased

No evident deposits of thorotrast.

rum analysis (performed at the

presence of thorium in the liver.

In brief, a man of 64 years, who 21 years previously had had injection of *thorotrast* in connection with cerebral angiography. At autopsy thorotrast deposits were found in the liver, spleen, bone marrow and *lymph nodes*. In connection with the large foci of thorotrast in the hilus of the liver, a moderately differentiated adenocarcinoma had developed from the *intrahepatic bile ducts*. Metastases were found in the liver and in the *regional lymph* nodes. The patient died in secondary heart failure.

DISCUSSION

In both cases *the thorotrast* tumours were probably elicited by radiation from *thorium particles* deposited in the tissues. As regards the latter case, where there was cancer of the bile duct, importance attaches

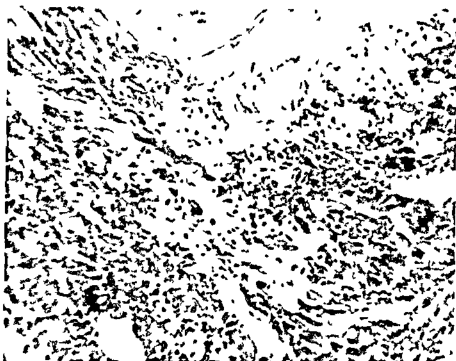


Fig 3
Case 2 Adenocarcinoma in the liver containing large deposits of thorotrast Dense masses in the lower portion represent the thorotrast (van Gieson) × 167

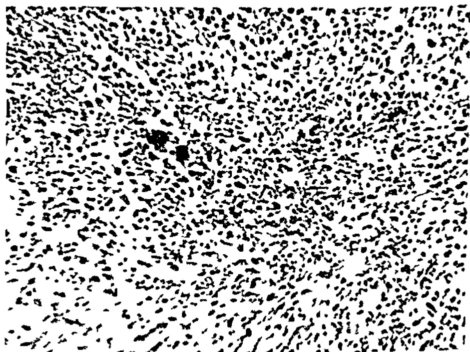


Fig 4
Case 2 Metastasis in the liver showing intermixture of carcinoma and thorotrast (van Gieson) × 167

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to the low incidence of such tumours in man in the Scandinavian countries. According to *Ringertz & Ericsson* (1961) only 0.7 per cent in Sweden.

The question of the carcinogenic action of thorotrast has been thoroughly discussed among radiologists, pathologists, radiophysicists and others. Many, including *Baserga et al.* (1960), consider that the number of cases of such tumours is definite proof of the carcinogenic effect of thorium. Others point to the fact that in spite of the large numbers of thorotrast injections given over many years, only about 60 cases have been reported or even noticed, owing to various circumstances such as absence of autopsy, and the fact that the physician was unaware that thorotrast had been used. That rather few cases of malignant thorotrast tumours have been described is certainly due in some degree to the long latency period. Radiographic examination involving thorotrast have probably been performed most frequently on patients who were elderly or seriously ill, many of whom die from old age or disease long before the tumours are due to appear. If all the patients receiving thorotrast had been followed for 20 to 30 years many more such tumours would certainly have been recorded. This is being increasingly realized and follow-up studies are being carried out in various parts of the world. It is therefore important that all cases of thorotrast tumours are recorded so that, with the information so obtained, we can widen our knowledge of the carcinogenic action of thorotrast.

SUMMARY

In connection with the reporting of two new cases of thorotrast tumours a survey of the literature in the field has been made which includes 59 cases. The latency period between the injection of the contrast medium and the appearance of the malignant tumour is about 18 years.

Two cases described are of a neurofibrosarcoma in the soft tissues of the neck, appearing in a 40 year-old man, 19 years after angiography with thorotrast, and a cholangiocellular carcinoma, which appeared in a 64 year old man 21 years after cerebral angiography with thorotrast.

ADDENDUM

Since this paper was submitted two more cases of malignant tumours following administration of thorotrast have been published.

1. *A. Nettleship* and *W. Fink* describe a hemangioendothelioma of liver in a 54 years old man who 16 years before death received an intravenous injection of 75 cc thorotrast. (Am. J. Clin. Path. 35, 422–426, 1961.)

2. *J. Suckow, G. Henegar* and *R. Baserga* report a case with a bile duct carcinoma of liver appearing 15 years after an intravenous injection of thorotrast. Amount of thorotrast is not mentioned. (Am. J. Path. 38, 663–670, 1961.)

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FROM UNIVERSITETETS INSTITUTT FOR GENERELL OG EKSPERIMENTELL PATOLOGI RIKSHOSPITALET OSLO (HEAD LEIV KREYBERG M.D.)

CELL METABOLISM IN EXPERIMENTAL SKIN CARCINOGENESIS

2. *Effects of a Single Application of Different Concentrations of 3-Methylcholanthrene (MCA) in Benzene Solution on the Rate of Formazan Deposition in the Epidermis of Hairless Mice*

By

OLAV HILMAR IVERSEN

Received 24. iii. 61

In previous papers (*Iversen* 1959, 1960) it has been shown that it is possible by a tetrazolium reduction method to estimate changes in the rate of formazan deposition in the epidermis of hairless mice after application of benzene and some polycyclic hydrocarbons, and also after roentgen irradiation (*Iversen & Devik* 1961). The amount of formazan deposited per mg dry epidermis per hour was measured at different time intervals after the initial application. The results were presented as the ratio between the values obtained in treated and in non-treated areas. The rate of formazan deposition was considered to be an expression of the endogenous dehydrogenase activity of the cells and thereby to be related to the rate of cell respiration.

A characteristic difference between the reaction type of the cells to some carcinogens (3-methylcholanthrene, 3,4,9–10 dibenzopyrene and 9,10 dimethyl 1,2 benzanthracene) on the one hand and to benzene and two non-carcinogenic compounds (phenanthrene and 1,2 benzanthracene) on the other hand, was observed. The three carcinogens produced a curve with an initial short rise in formazan deposition, followed by a depression of longer duration.

In the present paper the observations are extended to a study of the dose/response relationship after a local single application of 3 methylcholanthrene (MCA) in benzene solution.

MATERIAL AND METHODS

Six hundred and twenty one male and female mice were used. They were about 12 weeks old, weighed 16 to 28 grams and were kept on a standard diet. Varying doses of M.C.A. in benzene solution were applied to the skin of the mice in a given area by the use of a special pair of forceps as previously described (*Iversen* 1960).

The different solutions used in this study comprise 0.005 ml of: A. Benzene alone

B 1/128 per cent—, C 1/64 per cent—, D 1/32 per cent—, E 1/16 per cent— F 1/8 per cent—, G 1/2 per cent—and H 1/1 per cent MCA in benzene solution

At different time intervals (1 2 3 5,7 and 11 days) after the single application of the solutions the animals were sacrificed by fracturing the neck and their skin immediately flayed off The whole skin preparation was incubated in a tetrazolium solution and with the method of Cowdry the epidermis was stripped from the underlying skin in the treated area as well as in a control area from the opposite side of the back of the mouse The amount of formazan deposited per mg dry epidermis per hour was calculated with a photometrical method The ratio between the values from treated and non treated areas was considered to be an index of the effect of the application For a more detailed description of the method, reference is made to the previous papers

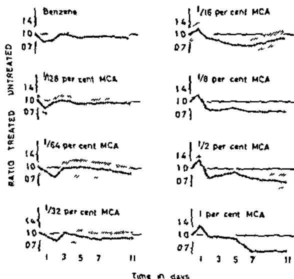


Fig 1

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RESULTS

Results are given in Fig 1 and Table 1 Values are calculated for groups of mice (see Table 1) at each of the above mentioned time intervals It appears that the reaction of the cells to the different experimental procedures may be grouped into two cathegories

(i) benzene alone and concentrations of MCA up to 1 32 per cent produce a slight initial fall in formazan deposition, while

(ii) concentrations of MCA from 1/16 per cent and up to 1 1 per cent produce the previously described characteristic curve with an initial rise in the rate of formazan deposition, followed by a decrease

TABLE I

The Mean Deviation from Normal Formazan Deposition in Epidermis after the Application of Benzene and Varying Doses of 3 Methylcholanthrene

Concentration	Days after application	No of mice	Arithmetic mean	SD of the mean
Benzene	1	12	0 801	0 07
	2	7	0 841	0 10
	3	8	1 000	0 12
	5	9	0 924	0 07
	7	7	0 964	0 10
	11	7	0 923	0 12
1/128 per cent 3 methylcholanthrene	1	20	0 805	0 07
	2	14	0 964	0 06
	3	16	1 022	0 09
	5	24	0 957	0 06
	7	16	0 979	0 06
	11	16	0 979	0 05
1/64 per cent 3 methylcholanthrene	1	12	0 885	0 08
	2	8	0 761	0 04
	3	8	0 972	0 10
	5	16	1 039	0 13
	7	8	0 994	0 11
	11	20	0 865	0 08
1/32 per cent 3 methylcholanthrene	1	20	0 911	0 06
	2	16	0 828	0 05
	3	16	1 010	0 11
	5	15	0 955	0 10
	7	16	0 884	0 09
	11	15	0 950	0 09
1/16 per cent 3 methylcholanthrene	1	12	1 161	0 13
	2	7	0 933	0 11
	3	8	0 878	0 06
	5	8	0 775	0 07
	7	14	0 764	0 07
	11	14	0 915	0 07
1/8 per cent 3 methylcholanthrene	1	12	1 1[illegible]5	0 08
	2	16	0 819	0 08
	3	15	0 791	0 07
	5	8	0 847	0 05
	7	8	0 774	0 05
	11	16	0 774	0 05
1/2 per cent 3 methylcholanthrene	1	16	1 278	0 11
	2	15	0 798	0 05
	3	15	0 857	0 05
	5	16	0 [illegible]44	0 05
	7	15	0 807	0 07
	11	8	0 756	0 0[illegible]
1 per cent 3 methylcholanthrene	1	16	1 261	0 05
	2	9	0 947	0 10
	3	11	0 980	0 06
	5	11	0 [illegible]9	0 04
	7	12	0 [illegible]6	0 07
	11	13	0 722	0 08

The relation between dose and initial response is shown in Fig 2 and Table 2 where the approximate integral of the first oscillation of the curve is related to dose Benzene and concentrations of MCA up to 1/32 per cent reveal integrals of about 0 700, while higher concentrations of MCA reveal integrals of about 1 120 to 1 200

The fundamental observation is thus that a critical dose level exists at which single applications of MCA changes the rate of formazan deposition in epidermis from the reaction type characteristic for benzene to the reaction type characteristic for sufficiently high concentrations of some carcinogens

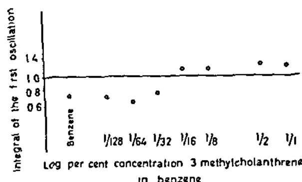


Fig 2

The relation between dose of MCA and initial response The ordinate gives the mean approximative integral of the first oscillations of the curves depicted in Fig 1 the abscissa indicates the different concentrations of MCA on a Log scale

TABLE 2

Relation between Dose and Response

Dose	Approximative integral of the first oscillation of the curves
Benzene	0 701
1/128 per cent 3 methylcholanthrene	0 707
1 64 per cent 3 methylcholanthrene	0 641
1 32 per cent 3 methylcholanthrene	0 742
1/16 per cent 3 methylcholanthrene	1 121
1/8 per cent 3 methylcholanthrene	1 124
1/2 per cent 3 methylcholanthrene	1 209
1 1 per cent 3 methylcholanthrene	1 196

DISCUSSION

The critical dose level observed in this study seems to reflect the situation at the production of tumours by *single* application of carcinogenic hydrocarbons According to *Terracini, Shubik & Della Porta* (1960) small single doses of a carcinogen do not give rise to tumours,

TABLE 1

The Mean Deviation from Normal Formazan Deposition in Epidermis after the Application of Benzene and Varying Doses of 3 Methylcholanthrene

Concentration	Days after application	No of mice	Arithmetic mean	SD of the mean
Benzene	1	12	0.801	0.07
	2	7	0.841	0.10
	3	8	1.000	0.12
	5	9	0.924	0.07
	7	7	0.964	0.10
	11	7	0.923	0.12
1/128 per cent 3 methylcholanthrene	1	20	0.805	0.07
	2	14	0.964	0.06
	3	16	1.022	0.09
	5	24	0.957	0.06
	7	16	0.979	0.06
	11	16	0.979	0.05
1/64 per cent 3 methylcholanthrene	1	12	0.885	0.08
	2	8	0.761	0.04
	3	8	0.972	0.10
	5	16	1.039	0.13
	7	8	0.994	0.11
	11	20	0.865	0.08
1/32 per cent 3 methylcholanthrene	1	20	0.911	0.06
	2	16	0.828	0.05
	3	16	1.010	0.11
	5	15	0.955	0.10
	7	16	0.884	0.09
	11	15	0.950	0.09
1/16 per cent 3 methylcholanthrene	1	12	1.161	0.13
	2	7	0.933	0.11
	3	8	0.878	0.06
	5	8	0.775	0.07
	7	14	0.764	0.07
	11	14	0.915	0.07
1/8 per cent 3 methylcholanthrene	1	12	1.165	0.08
	2	16	0.819	0.08
	3	15	0.791	0.07
	5	8	0.847	0.05
	7	8	0.774	0.05
	11	16	0.774	0.05
1/2 per cent 3 methylcholanthrene	1	16	1.278	0.11
	2	15	0.798	0.05
	3	15	0.857	0.05
	5	16	0.944	0.05
	7	15	0.807	0.07
	11	8	0.756	0.09
1 per cent 3 methylcholanthrene	1	16	1.261	0.05
	2	9	0.947	0.10
	3	11	0.980	0.06
	5	11	0.969	0.04
	7	12	0.696	0.07
	11	13	0.722	0.08

FROM THE UNIVERSITY INSTITUTE FOR EXPERIMENTAL MEDICINE COPENHAGEN Ø DENMARK HEAD JENS BING M D

DEPOSITION OF SERUM PROTEINS IN VASCULAR WALLS DURING ACUTE HYPERTENSION

By

JOHN GIESE

Received 7 ix 61

In 1933 *Schurmann & MacMahon* advanced the theory, that an infiltration of plasmatic constituents into the vascular wall is a very important factor in the pathogenesis of hypertensive vascular disease (4) This conception has received support from many investigators (review by *Zollinger* (6)), but another theory favours necrosis of the media as the principal factor (e g *Montgomery & Muirhead* (2))

It was felt, that some information concerning the possible deposition of serum proteins in vascular walls during acute hypertension might be obtained by injecting experimental animals with homologous serum proteins, conjugated with a fluorescent tracer dye, followed by the administration of a pressor agent The following report gives the results of such studies in rats exposed to repeated rises in blood pressure, caused by injections of synthetic angiotensin, as compared with normal control animals

MATERIAL AND METHODS

14 female albino rats were used (average weight 200 grammes)

Pooled rat serum was conjugated with Lissamine Rhodamine B 200 (G T Gurr) as described by [illegible]

d [illegible] mental set up was [illegible] equal amounts of [illegible] mes (1 4 ml) with [illegible] mg/ml) being employed One rat was then given repeated intravenous injections of a solution of synthetic angiotensin, the other serving as a control received injections of 0 9 per cent saline solution Injections

This work was [illegible]

within a certain, narrow dose range the tumour yield increases step like and with still higher doses there is only a very small further effect. The conclusion of the last mentioned paper is "A critical dose level exists at which single application of carcinogens becomes fully effective".

The observation presented in this paper, thus may give some further support to the hypothesis that the characteristic curve profile observed *with the described method is related to the carcinogenic property of* the compounds. Further studies, involving a "blind test" of 20 different carcinogens, cocarcinogens and non-carcinogenic compounds, are in progress.

SUMMARY

i. A tetrazolium reduction method is used to measure the amount of formazan deposited per mg dry epidermis per hour in hairless mice after the local, single application of 0.005 ml of different concentrations of MCA in benzene solution.

ii. A critical dose level exists at which MCA changes the rate of formazan deposition from the reaction type characteristic for benzene and to the reaction type previously described as characteristic for sufficiently high doses of some carcinogens.

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Terracini B., Shubik Ph. & Della Porta G. A Study of Skin Carcinogenesis in the Mouse with Single applications of 9,10 Dimethyl 1,2 benzanthracene at Different Dosages. Cancer Res. *20*: 1538–1541, 1960.

In animals treated with angiotensin as well as in control animals fluorescence could be seen in the lumina of blood vessels, where residual plasma was present in the sections The Kupffer cells of the liver generally showed rather strong fluorescence

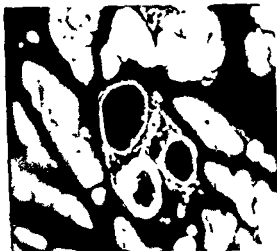


Fig 1
Small pancreatic artery sh wing fluorescence located in the media In this black and white ph tographs the light emitted by the fluor chrome is depicted as white, under the micr scope an range red fluorescence is seen (× 175)

In 6 of 8 animals injected with angiotensin coloured deposits of the fluorescent protein conjugate were found in the walls of arterial vessels In 5 of these animals the vessels of the pancreas were affected (Fig 1), deposits were also found in the walls of arterial vessels in the intestinal wall or in the mesenterium (5 animals) In 2 cases an artery in the right side of the heart w s affected No deposits have been found in renal or pulmonary vessels, nor in the aorta or periaortic vessels

The vessels showing these deposits were small arteries and arterioles, the diameter of some of these vessels was measured and found to vary between approximately 25 µ and 75 µ Only vessels showing convincing deposits in the media were counted as positive, the deposits were sometimes seen in the whole, sometimes only in a part of the circumference In some cases a vessel could be followed in 5–6 consecutive sections, and it was observed that varying parts of the circumference were involved in the various sections

Often only one of several vessels visible in a section showed fluorescent medial deposits, and such affected vessels were sometimes found in only one or two of several slides prepared from the organ in question, but in a single animal many vessels showing fluorescent deposits were found in nearly every section of the pancreas

were given (almost) simultaneously in the two animals, volume of doses number of injections and intervals between injections were kept equal The angiotensin solution was prepared by dissolving 0 5 mg of synthetic angiotensin in 5 ml 0 9 per cent saline the volume injected was varied doses of 0 1 ml (10 microgrammes) were often used Injections were given every 2 3 4 or 5 minutes, the next injection was usually given when the blood pressure had returned or nearly returned to the baseline About 40 50 injections were usually given total time of experiments was 2 4½ hours In two cases no control animal was prepared

The rats were killed by exsanguination at the termination of the experiment Post mortem examination was performed and organs fixed in formalin buffered with calcium acetate Paraffin sections were prepared Deparaffinized sections were examined under a Reichert Zetopan microscope using ultraviolet blue radiation with primary and secondary filters as described by *Chadwick McEntegart & Nairn* (1)

Several sections were prepared from the pancreas and small intestine sections of the heart kidney liver spleen lung and aorta were also examined Care was taken to examine a comparable number of sections from control animals and angiotensin treated animals

In some cases sections were examined under the fluorescence microscope and vessels showing fluorescent deposits in the walls were located on the slide by coordinates established with a graduated mechanical microscope stage, a sketch of the vessel was drawn showing the location of the deposits in the wall Afterwards these sections were stained with hematoxylin eosin the periodic acid Schiff procedure or the Feulgen reaction The sections were reexamined by conventional light microscopy and the vessels relocated by use of the coordinates

RESULTS

Blood Pressure Recordings

In the first experiments it was tried to administer angiotensin as a continuous infusion a primary rise in blood pressure was soon followed by a decrease (for instance a rise of 45 mm decreasing in approximately 10 minutes to a basic value of 110 mm during the infusion of about 14 microgrammes of angiotensin per minute per kg) Since the primary aim was to obtain large rises in blood pressure this technique was abandoned and the procedure involving repeated injections adopted In 2 of 8 angiotensin treated rats experiments were started with a period of continuous infusions but continued with repeated single injections In 6 animals the latter procedure was employed exclusively

It was found necessary to employ separate syringes and polyethylene tubes for the injections of the angiotensin solutions because it is difficult to remove all angiotensin at least by simple flushings with water

Blood pressure recordings were performed in 7 of 8 angiotensin treated animals repeated rises in blood pressure were observed No recordings have been performed in the control animals At the beginning of an experiment rises of 40 50 mm of mercury were often seen most often a decreasing response was observed in the run of the experiment It was noted that when intervals between injections were lengthened (as in example 5 min instead of 3 min) the response to the same dose would often increase Furthermore the response was varying in different animals

Fluorescence Microscopy

The fluorescent dye used for the conjugation with serum proteins shows a deep orange fluorescence with very good contrast to the autofluorescence of tissues

is shown, that an infiltration into the vascular wall of a substance, originating from the blood plasma, can take place under the experimental conditions described

Whether these deposits are due to a primary, isolated change in the permeability of the vessel wall, or they are the expression of secondary permeability changes, caused by more gross vascular damage, is not known

As a method for the study of the pathogenesis of acute hypertensive vascular damage, the experimental approach used here is obviously open to several criticisms Synthetic angiotensin was used in very high doses Furthermore, serum proteins coupled to a fluorescent dye were injected, such conjugated proteins may not behave completely like native proteins The load imposed upon the reticuloendothelial system (as evidenced by the uptake of tracer protein by the Kupffer cells) might also be of importance, *Zweifach* has demonstrated, that blockade of the reticuloendothelial system may be accompanied by important changes in vascular reactivity and vulnerability (7)

Whether the findings reported bear any relevance as to the understanding of the pathogenesis of hypertensive vascular disease in the usual sense of the word, cannot be settled now But in favour of the concept of protein infiltration as a factor in the pathogenesis it may be mentioned, that *Ohta et al* by means of immuno histological technique have been able to demonstrate deposits of gamma globulin in damaged arteries in the early stages of desoxycorticosterone-induced hypertension in the rat (3)

SUMMARY

Rat serum proteins, conjugated with a fluorescent tracer dye, were injected into rats Some of these rats were exposed to repeated injections of synthetic angiotensin with resultant repeated rises in blood pressure 6 of 8 rats showed deposits of fluorescent protein tracer in arterial or arteriolar walls pancreatic and intestinal vessels being most often involved These deposits were clearly PAS-positive In 6 control rats exposed to repeated injections of saline, no such deposits were found

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It was tried to correlate these findings with the blood pressure responses observed in the various animals, the impression was gained, that animals showing large rises in blood pressure were more likely to develop deposits, but no safe conclusions could be drawn

A comparable number of sections prepared from the organs of 6 control rats was examined, no fluorescent deposits in arterial or arteriolar walls were found in any of these sections



Fig 2

Section of pancreas (PAS stain) showing a small artery with PAS positive material in the media only a part of the circumference is involved (× 250)

Conventional Light Microscopy

As mentioned previously, some vessels showing fluorescent deposits in the wall were located under the fluorescence microscope and afterwards relocated under conventional light after staining by various procedures The fluorescent deposits were found to be clearly PAS-positive (Fig 2) exact correspondence was noted between the localization of fluorescent protein tracer and the PAS positive deposits In hematoxylin-eosin and Feulgen-stained sections a vacuolated structure of the media was sometimes observed In some vessels a loss of medial nuclei was noted in others a darker staining of nuclei together with an angular or shrivelled appearance was observed

DISCUSSION

The experiments reported here show, that deposits of serum protein tagged with a fluorescent tracer dye, can be found in arterial and arteriolar walls of animals, which have been exposed to repeated rises in blood pressure These deposits were found to be PAS-positive Thus it

THE SENSITIVITY OF N. GONORRHOEAE TO ANTIBIOTICS

By

OLOF RINGERTZ

Received 20 1 1961

The sensitivity of N gonorrhoeae to sulphonamides and antibiotics has been extensively studied Reduced sensitivity to sulphonamides has been reported by many authors (*Cohn & Seijo* 1943, *Del Love & Finland* 1955) Though most authors earlier denied the existence of strains resistant to penicillin (*Lodin* 1955, 1956, *Del Love & Finland* 1955 and *Finland* 1955, 1958) there are some recent reports of reduced sensitivity to this drug (*Thayer et al* 1957, *Cradock-Watson et al* 1958, *Reyn et al* 1958 and *Hirsch, Finland & Wilcox* 1960) Strains highly resistant to penicillin have not been observed Though streptomycin has not been much used in the treatment of gonococcal infections recent reports indicate increased resistance to this antibiotic (*Alergant* 1958, *Cradock-Watson et al* 1958) The sensitivity of N gonorrhoeae to tetracycline has been studied by *Gocke, Finland & Wilcox* 1950, by *Del Love & Finland* 1955 and by *Hirsch, Finland & Wilcox* 1960 So far no tetracycline resistant strains have been reported

During the last few years the number of requests for sensitivity determination of gonococcal strains has increased in this laboratory Many physicians in Sweden have experienced an increased number of therapeutic failure with the usual dosage of one injection of 600,000 IU of procain penicillin Similar experience have also been frequently reported from other countries (*Franks* 1946, *Duemling & Horton* 1947, *Thayer et al* 1957, *Willcox* 1957, *Reyn et al* 1958 and *Gjessing* 1959) The object of this study has been to determine how frequent strains with reduced sensitivity to certain antibiotics are in Sweden

METHODS

The specimens were sent to the laboratory in a modified Stuart medium (*Ringertz* 1960 and were cultured on a heated blood agar medium of the following composition

6600 ml 1 7 per cent nutrient agar pH 7 2 (2 l H_2O/1000 g meat, 1 per cent peptone 0 3 per cent NaCl 0 2 per cent Na_2HPO_4 17 g agar/l) 500 ml horse blood 600 ml horse serum

... [illegible] ...iteria had to be fulfilled
... [illegible] ...menting dextrose but not

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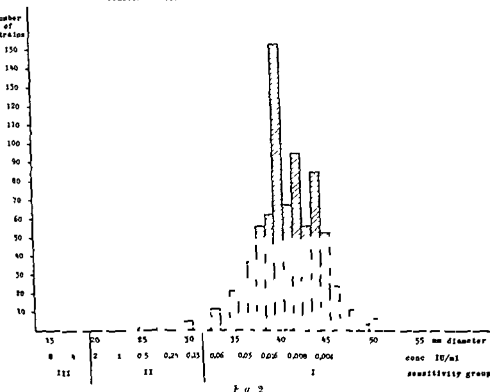


Fig 2

The strains studied were divided into 4 different sensitivity groups according to Table 1 suggested by *Ericsson et al*

MATERIAL

All strains isolated in routine diagnostic work over a period of 6 months in 1959 and 1960 a total of 809 were included in the study from patients with a positive culture from more than one locality only one strain was included. In cases where repeated cultures were positive new determinations of the sensitivity were made on each occasion. Thus the 809 strains were isolated from 777 patients.

The 32 strains obtained from 26 patients by repeated culture after treatment were recorded separately. If the inhibition zones for penicillin or sulphonamide were [illegible] mm or for streptomycin [illegible] 2) mm the test was repeated. The two determinations gave roughly the same results. Thus for penicillin 13 strains were found in sensitivity group II in the first test and I in the second. For the strains highly resistant to sulfa or streptomycin there was no difference between the two recordings. Only the values obtained in the first test were included in the material.

RESULTS

The results are shown in Figures 1 to 4

As can be seen in Figure 1 the majority of the strains (99.0 per cent) were highly sensitive to *sulphonamide* most of them inhibited by 0.5

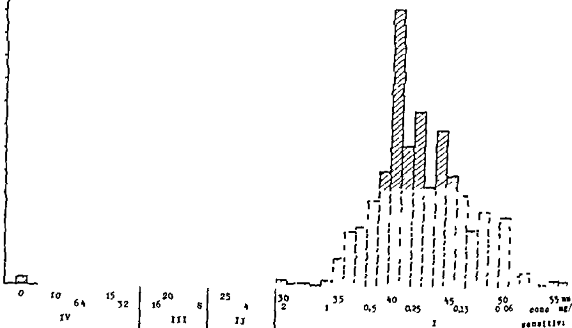


Fig 1

Sensitivity Tests

The sensitivity tests were performed with the paper disc method reported by Ericsson et al 1954 and 1960 on a medium identical with that used for the culture except that the peptone was excluded Paper discs prepared at the Bacteriological Department of Karolinska sjukhuset Stockholm were used The chemotherapeutic content of the different discs was sulphonamide (Elkosin Ciba) 2 4 mg penicillin (Benzyl penicillin Kabi) 20 IU streptomycin (Strepsulfat Kabi) 50 mcg and tetracycline (Tetracycline Lederle) 50 mcg

The test was carried out on 10 cm plates with a medium layer of approximately 5 mm The plate was flooded with a bacterial suspension made with about 20 colonies of gonococci in 3 ml broth The plate was allowed to dry for 10 minutes and the paper discs were then placed on the surface of the medium Diffusion was allowed for 3 hours at room temperature prior to incubation at 37° C in 10 per cent CO_2 + air for 24 hours The diameters of the zones were measured and the corresponding minimal inhibitory concentrations calculated according to Ericsson et al 1954 and 1960 As a control staphylococcus aureus FDA 209 was run simultaneously

TABLE 1

Values of Inhibiting Concentration in vitro

Group	Characterization	Sulphonamide mg 100 ml	Penicillin IU 100 ml	Streptomycin mcg ml	Tetracycline mcg ml
I	Sensitive	< 2 5	< 0 1	< 4	< 1
II	Fairly sensitive	2 5 7 5	0 1 2	4 16	1 4
III	Slightly sensitive	7 5 25	2 20	16 100	4 50
IV	Resistant	> 25	> 20	> 100	> 50

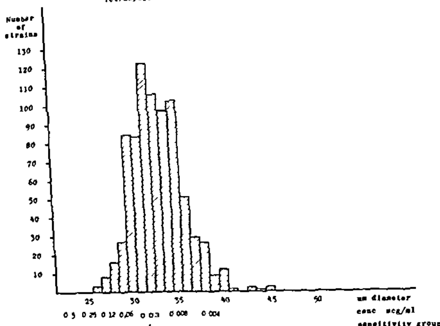


Fig. 4

In four of the nine cases yielding gonococci of reduced sensitivity to streptomycin the source of infection could be traced to South America. The rest of the cases had been infected in Sweden.

DISCUSSION

0.4 per cent of the strains tested were found to be highly resistant to sulphonamides. This frequency is low compared to earlier results by other authors (*Gocke, Wilcox & Finland* 1950) but similar to the results reported by *Cradock-Watson et al.* A decreasing frequency of gonococcal strains resistant to sulphonamides, which may be due to the fact that sulphonamides have not been in general use for the treatment of gonorrhoea for the last 15 years, has been noticed by *Finland* (1955, 1958, 1960).

Some strains with reduced sensitivity to penicillin were found in this material. These strains showed the same degree of resistance as reported by *Reyn et al.* 1958 and *Cradock-Watson et al.* 1958 but the frequency was notably lower than reported by these two groups. No strain was found to be highly resistant to penicillin.

The number of strains resistant to streptomycin was also lower than reported by the two groups mentioned above.

The relationship between reduced sensitivity to streptomycin and

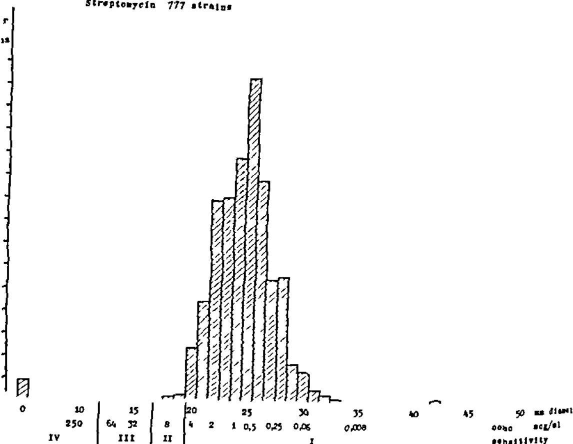


Fig 3

mg% or less The remaining 1 0 per cent showed a reduced sensitivity, 3 of them being highly resistant (0 4 per cent)

For *penicillin* (Fig 2) the distribution is somewhat different No highly resistant strains were found The majority were referred to sensitivity group I, being inhibited by 0 1 IU/ml, and 1 7 per cent of the strains to sensitivity group II

97 9 per cent of the strains were sensitive to *streptomycin* (Fig 3), being inhibited by 4 mcg ml or less The remainder showed a reduced sensitivity, the majority of them (1 2 per cent of the total number) being highly resistant to streptomycin

All strains were sensitive to tetracycline, being inhibited by 0 25 mcg/ml or less (Fig 4)

Strains resistant to streptomycin often showed a reduced sensitivity to penicillin as well Thus, out of the 9 strains highly resistant to streptomycin one strain required 0 5 IU ml penicilling for inhibition, five 0 13 IU/ml and three 0 05 IU/ml

Three of the 32 strains obtained at repeated culture after treatment were referred to sensitivity group II for penicillin, while 5 were resistant to streptomycin

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penicillin, the tracing of some of the streptomycin resistant strains to the same source and the fact that all nine such strains appeared within a period of 3 months in 1960 (5 of the cases within 18 days) indicate that the strains are epidemiologically stable and have good possibilities to spread

SUMMARY

The sensitivity of 809 strains of N gonorrhoea to sulphonamides penicillin, streptomycin and tetracycline was studied with the aid of a paper disc method

1 1 per cent of the strains showed reduced sensitivity to sulphonamides,
2 1 7 per cent showed a slightly reduced sensitivity to penicillin No highly resistant strains were found
3 2 per cent of the strains showed reduced sensitivity to streptomycin Nine strains (1 2 per cent) were highly resistant Four of these nine cases were found to have been infected by a common source
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SUMMARY

The sensitivity of 809 strains of N gonorrhoea to sulphonamides, penicillin, streptomycin and tetracycline was studied with the aid of a paper disc method

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STUDIES ON THE COMPLEMENT ACTIVITY IN THE TREPONEMA PALLIDUM IMMOBILIZATION (TPI) TEST

A Comparative Study of the Immobilizing and Hemolytic Complement Activity

By

BENGT HEDERSTEDT

Received 1 ii 1961

In the routine TPI test, an excess of hemolytic activity is the only criterion of the usability of a particular complement (*i e* native guinea pig serum) But it has not been made clear whether the complement activity that is operative in the immobilization of treponemes may vary between different complements or whether any *definite relationship* exists between the immobilizing and hemolytic activities of a complement These problems are of theoretical interest and also have a practical bearing on, inter alia, the sensitivity of the TPI test itself and on the reproducibility of quantitative TPI tests The immobilizing and *hemolytic activities of different complements under standard conditions* have therefore been determined and compared in this study

MATERIALS AND METHODS

Determination of Immobilizing Complement Activity

The immobilizing activity of the complement was determined by means of quantitative TPI tests on a known TPI positive control serum The resulting titre was used as measure of the immobilizing activity of the complement

The TPI test was performed by [illegible] (1949) and *Nelson & Diesendruck* (1951) with th[illegible] basal medium *the total concentration of sodium* [illegible]lutathione 0 08 per cent Ultrafiltrate of beef seru[illegible]nt and treponemal suspension were mixed in the ratio 10 40 50 in a total quantity of 0 5 ml

Complement The complements consisted of guinea pig sera They were kept in suitable portions in a freezer at about —60° C A small quantity of each complement was inactivated (56° C 30 mins) and was used as inactive complement in controls

Positive control serum TPI positive control serum was *prepared from a mixture* of two sera from patients with tertiary syphilitic symptoms The serum mixture had the same titre as control serum no III prepared by WHO Reference Centre in Copenhagen Control serum was diluted with saline in two step dilution from 1/1 to 1/256 Pipett was changed for each step For all tests in one series the control serum was diluted once and for all the batches with the diluted serum being kept at —20° C dispensed in small portions so as not to be thawed more than once

Negative control serum TPI negative control serum consisted *of a mixture of two* sera from healthy blood donors

Basal medium Each batch of basal medium was prepared in a quantity sufficient for all tests in one series It was kept at —20° C in small portions so as not to be thawed more than once The reducing capacity of the 1.5 per cent sodium thioglycollate solution in the basal medium was estimated with iodine solution At least 90 per cent of the theoretically reducing potency was required for use in the tests

Rabbits The testicles of X ray treated rabbits (total 600 r) were collected 6–8 days after the inoculation

Treponemal suspension The number of treponemes in 0.005 ml of treponemal suspension under a 18 × 18.2 mm cover glass at 500 × was about five per microscopic field (c 4.5 × 10[6] treponemes per ml)

Controls The following controls were included in every test: 1) and 2) treponemal suspension plus active and inactive complement, respectively, 3) and 4) TPI negative controls serum with treponemal suspension plus active and inactive complement, respectively In quantitative TPI tests TPI positive control serum was tested only undiluted with treponemal suspension plus inactive complement At least 80 per cent survival was required in the controls

In addition qualitative controls for excess of hemolytic activity of the complement were made on all tubes by adding 0.1 ml of 2 per cent suspension of sensitized sheep blood cells 100 per cent hemolysis was required for evidence of hemolytic excess

Incubation and reading Incubation was done at 35° C for 18 hours At least 50 …

… plus inactivated complement

Determination of Hemolytic Complement Activity

The hemolytic activity of the complement was determined by the method of Kabat and Mayer (1948) The total volume of the lytic system was 1.25 ml The complement dilution was 1 100 The volumes of the various reagents are given in Table 1

TABLE 1

Complement Titration Schedule

Tube no	1	2	3	4	5	6	7	8	9
Veronal NaCl buffer ml	0.70	0.65	0.60	0.55	0.50	0.45	0.40	0.35	0.30
Complement dilution ml	0.05	0.10	0.15	0.20	0.25	0.30	0.35	0.40	0.45
Sensitized sheep erythrocytes	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

Sheep blood The sheep blood with addition of ACD solution (1 part ACD solution to 4 parts blood) and EDTA (5 ml 0.15 mol Na_2 EDTA …

… every titration the percentage of hemol… For 50 per cent unit of the complement wa… the equation the titre being indicated in log units …ough a

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MATERIALS AND METHODS

Determination of Immobilizing Complement Activity

The immobilizing activity of the complement was determined by means of quantitative TPI tests on a known TPI positive control serum. The resulting titre was used as measure of the immobilizing activity of the complement.

The TPI test was performed by the technique of *Nelson & Mayer* (1949) and *Nelson & Diesendruck* (1951) with the following modifications. In the basal medium the total concentration of sodium thioglycollate was 0.18 and of glutathione 0.09 per cent. Ultrafiltrate of beef serum was excluded. Serum, complement and treponemal suspension were mixed in the ratio 1.0 : 4.0–5.0 in a total quantity of 0.5 ml.

Complement. The complements consisted of guinea pig sera. They were kept in suitable portions in a freezer at about −60° C. A small quantity of each complement was inactivated (56° C, 30 mins.) and was used as inactive complement in controls.

Positive control serum. TPI positive control serum was prepared from a mixture of two sera from patients with tertiary syphilitic symptoms. The serum mixture had the same titre as control serum no. III prepared by WHO Reference Centre in Copenhagen. Control serum was diluted with saline in two step dilution from 1/1 to 1/256. Pipett was changed for each step. For all tests in one series the control serum was diluted once and for all, the batches with the diluted serum being kept at −20° C dispensed in small portions so as not to be thawed more than once.

Negative control serum. TPI negative control serum consisted of a mixture of two sera from healthy blood donors.

TABLE 3

Analysis of Variance in Reproducibility Tests

	Variation	Sum of squares	Degrees of freedom	Mean squares S^2	V^2 test	Standard deviation σ	$3\sqrt{2}\,\sigma$
Immobilizing activity	Between duplicate tests	0.011390	16	$S_1^2 = 0.000712$		0.027	0.113
	Between tests carried out on different days	0.061505	15	$S_2^2 = 0.004100$	$\frac{S_2^2}{S_1^2} = 5.76$	0.064	0.272
Hemolytic activity	Between duplicate tests	0.000570	16	$S_1^2 = 0.000036$		0.006	0.025
	Between tests carried out on different days	0.011100	15	$S_2^2 = 0.000740$	$\frac{S_2^2}{S_1^2} = 20.6$	0.027	0.113

EXPERIMENTAL

This study consisted chiefly of reproducibility experiments and of determinations of the immobilizing and hemolytic activities of different complements

Reproducibility experiments The reproducibility was studied by means of immobilizing and hemolytic activity tests made on the same day and on different days

On the same complement, duplicate tests of immobilizing and hemolytic activity were made om sixteen separate days in the course of five weeks *For each test of hemolytic activity (including duplicate tests* made on the same day) a new complement dilution was prepared, and for each day of testing, treponemes from different rabbits and new blood cell dilutions from the same sheep blood were used for determination of the immobilizing and hemolytic activities

The immobilizing activity varied in the 32 tests between 2 787 and 2 554 (difference 0 233), with mean value 2 664 The hemolytic activity varied between 2 903 and 2 839 (difference 0 064), with mean value 2 874 (Table 2)

TABLE 2
Reproducibility Experiments

	Number of days of testing	Number of tests	Mean activity	Deviations from mean max	min
Immobilizing activity	*16*	*32*	*2 664*	*0 123*	*0 002*
Hemolytic activity	16	32	*2 874*	0 035	0 002

In duplicate tests the greatest difference in immobilizing and hemolytic activity was 0 069 and 0 020, respectively Analysis of variance (*Hald* 1952) was carried out to establish whether there was any difference in reproducibility of duplicate tests made on the same day and of tests *made on different days The mean square for duplicate tests* was 0 000712 and 0 000036 for the immobilizing and hemolytic activities, respectively For tests made on different days the corresponding figures were 0 004100 and 0 000740 The analysis data are given in Table 3

The variance ratio $\frac{S}{S_r}$ for the immobilizing and hemolytic activities was 5 76 and 20 6, thus differing significantly from 1 in both cases

The standard deviation in duplicate tests was 0 027 and 0 006 and *for tests on different days 0 064 and 0 027, for immobilizing* and hemolytic activities, respectively Thus, if $3\sqrt{2}\sigma$ is chosen as level of significance, an immobilizing and hemolytic activity of 0 113 and 0 025 in duplicate tests will be significantly greater than zero The corresponding difference in activity for tests on different days is 0 272 and 0 113 (*Table 3*)

The reproducibility in these tests appears to be good In *Nielsen's*

TABLE 4

Determination of Hemolytic and Immobilizing C' Activity on Serum from 16 Guinea Pigs

Guinea pig serum no	Hemolytic C activity log 50 per cent units	Immobilizing C -activity log TI I_{50}
1	2.611	2.605
2	2.721	2.781
3	2.721	2.826
4	2.522	2.721
5	2.721	2.777
6	2.620	2.739
7	2.623	2.684
8	2.674	2.806
9	2.682	2.699
10	2.586	2.911
11	2.551*	2.595
12	2.509	2.692
13	2.522	2.649
14	2.647	2.696
15	2.620	2.705
16	2.824	2.583

* In qualitative test for excess of hemolytic activity this serum did not yield complete hemolysis

luated if they recurred in repeated tests But the serum from one guinea-pig did not suffice for more than one or at most two tests For several tests to be made with the same complement, therefore, pools of complements would have to be used

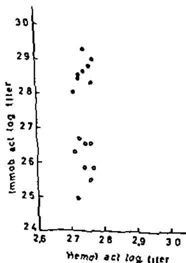


Fig 2

Relation between immobilizing and hemolytic activities of two complements (A and B) in eight tests

C'A ●   C'B ○

study (1957), for example, the variations between duplicate tests of immobilizing activity were about four times higher in preliminary experiments and about ten times higher in the main experiments. The better reproducibility in the present study may be ascribed principally to the greater degree of standardization of the method.

Comparison between immobilizing and hemolytic activity of different complements. Sera from each of sixteen guinea-pigs were tested in the same experiment with respect to immobilizing and hemolytic activity of the complement. The complements were added to the tubes for determination of the immobilizing activity at the same time as the titration of the same complement was started for determination of hemolytic activity.

In the determination of immobilizing activity the reading lasted 3 hours, whereas for technical reasons the time of incubation could not be maintained constant for the different tubes but varied between 18 and 21 hours. According to, among others, *Portella & Thompson* (1957), the sensitivity of the TPI test is directly proportional to the square of the reaction time, which should be taken into account when assessing the results. For this reason the results are set out in Table 4 in the order in which the readings were made. The relation between immobilizing and hemotlytic activity is illustrated graphically in Fig. 1.

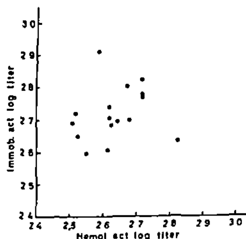


Fig. 1.
Immobilizing in relation to hemolytic C' activity in serum from 16 guinea pigs.

The immobilizing activities lay between 2.911 and 2.583, mean 2.717, and the hemolytic between 2.824 and 2.509, mean 2.635.

It is seen from Fig. 1 that the points for two of the 16 sera (Nos. 10 and 16 in *Table 4*) are noticeably separated from the remainder. Complement No. 10 had a significantly greater immobilizing but lesser hemolytic activity than complement No. 16. The difference was 0.328 and 0.238, respectively.

The differences in activity could, of course, be more reliably eva-

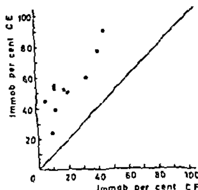


Fig 4
Relation between immob l ty percentages for ten sera determined with two c mplements E and F

lytic activity of the complement on six different days The results are shown in Fig 3

The immobilizing activity in the six tests varied between 2 878 and 2 736 for C C and between 2 630 and 2 475 for C D the means being respectively 2 819 and 2 553 The difference in activity varied between 0 331 and 0 225 with mean 0 266 In each test C C had a significantly greater immobilizing activity than C D

The hemolytic activity varied between 2 824 and 2 744 for C C and between 2 939 and 2 886 for C D means 2 779 and 2 918 The differences in activity varied between 0 177 and 0 115 mean 0 139 In all tests C C had a significantly lower hemolytic activity than C D

Thus in the three investigations no definite proportionality was found between immobilizing and hemolytic activity of the complement

Use of complements with different immobili ing activity in TPI test for routine diagnosis The following experiments were carried out to illustrate the importance of using complements with different immo bilizing activity in TPI tests for routine diagnosis

In the same TPI test determinations were made of the immobilizing capacity of ten different patient sera with two complements F and F The ten sera came from patients with untreated or treated primary syphilis and had a low titre in TPI tests C E and C F had *been simul* taneously tested in advance and been found to have an immobilizing *activity of 2 930* and *2 648* respectively with hemolytic activity of 2 886 and 2 903

Fig 4 illustrates the relationship between the immol lity percent ...

... C I and between 46 *and 6 with C F*

With C I the TPI test was positive (immobility per cent ≧ 50) in

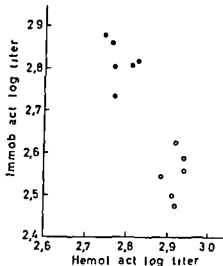


Fig 3

Relation between immobilizing and hemolytic activities of two complements C and D in five tests
C'C . C'D °

Two pools of complement, A and B, each consisting of serum from fifteen guinea-pigs selected at random, were tested for immobilizing and hemolytic activity on eight different days The results are shown in Fig 2

The immobilizing activity varied in the eight tests between 2 931 and 2 803 for C'A and between 2 670 and 2 493 for C'B The mean activities were 2 864 and 2 602 and the difference in activity varied between 0 330 and 0 174, mean 0 261 *C'A had a significantly greater immobilizing* activity than C'B in all tests

The hemolytic activity for C'A and C'B varied between 2 769 and 2 710, means 2 745 and 2 742 The difference in activity varied between + 0 012 (activity for C'A > C'B) and — 0 024 (activity for C'A < C'B), mean — 0 003 In no test was there a significant difference in hemolytic activity between C'A and C'B

Thus in this series two complement pools were tested, having the same hemolytic but significantly different immobilizing activity

The selection of two pooled complements, one having greater immobilizing but less hemolytic activity than the other (as complements Nos 10 and 16 in Table 4), was done as follows

Sera from each of a number of guinea-pigs were tested in order to obtain an approximate notion of their immobilizing and hemolytic activity without using a large quantity of complement Therefore the treponeme survival and the hemolysis rate were determined in only one tube containing a suitable TPI-positive control serum solution and dose of complement The quantity of guinea-pig serum used for this purpose was only about 0 25 ml Based on the results of this screening test two pools, C'C and C'D, were prepared from the remainder of the guinea-pig sera tested and were examined for immobilizing and hemo-

were not always fixed to the same degree by an immune precipitate This has later been verified and discussed by, among others, *Silverstein* (1954) It still remains to see, however, whether any parallel can be drawn between this observation and the difference found in the present study between the immobilizing complement activity of different guinea pig sera

Both *Nielsen* (1957) and *Portella & Thompson* (1957) discussed the relationship between hemolytic and immobilizing C activity and thought it probable that the relationship might vary from complement to complement Thus *Portella & Thompson* state "it is well known that for different preparations fixability should not be considered as necessarily proportional to hemolytic activity Sera from a fairly large number of guinea pigs are pooled with a view to making the averaged activities approximately proportional

This statement has not been confuted by the present study It is clear that pooled complement consisting of sera from a large number of animals must be used if good reproducibility is to be attained in quantitative TPI tests But it must be noted that a significant difference in immobilizing activity was found in this study between pools from 15 animals, and that in tests not reported here a significant difference was found between pools from up to 30 animals

The sensitivity of the TPI test for routine diagnosis is important It was found possible to enhance the sensitivity by using complement with highly immobilizing activity Preliminary experiments have shown that complement does not lose its immobilizing activity even after one year's storage at —60° C, so that it would appear that this selective procedure may prove applicable within routine diagnosis

SUMMARY

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of

No definite correlation was proved in these studies between immobilizing and hemolytic activity A comparison is given between two complements one of which had a significantly greater immobilizing but a significantly lower hemolytic activity than the other

Finally examples are shown of how the sensitivity of the routine TPI test was increased by the selection of complement with high immobilizing activity

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seven cases and slightly positive (49 – 20 per cent) in three, while with CF it was slightly positive in four cases and negative (19 – 0 per cent) in the remaining six

This experiment showed that it is possible to increase the sensitivity of the TPI test *through complement selection based on the immobilizing activity of the complement*

DISCUSSION

No definite proportionality has been found in this study between immobilizing and hemolytic activity of the complement The difference found between the immobilizing activities of different complements has been of *an order of magnitude that can be of importance in routine* diagnosis It seems natural to suppose that the difference in immobilizing activity between different complements, in analogy with the inactivation of certain viruses, phages and bacteria produced by native serum, may be due to the fact that different guinea-pig sera cause different degrees of non-specific immobilization of the treponemes In the majority of tests the survival was lower in the negative controls with active complement than in those with inactive But, as this lower survival was observed only with certain treponemal suspensions, and sometimes with both strongly and weakly immobilizing complement, it cannot be excluded that it was caused by sensitization of the treponemes *in vivo* *Further studies are required, however, to verify this* assumption With the object of preventing falsely elevated values in the immobilizing complement activity test as a result of this possible sensitization, the following precautions were taken In determining the immobilizing activity a calculation was made not of the number of motile treponemes in the tubes containing TPI-positive control serum plus active complement in relation to the number of motile treponemes in the control containing TPI-positive serum plus inactive complement but in relation to the number of motile treponemes in the control containing TPI negative serum plus active complement On the basis of this *calculations it cannot, however, be settled that the difference in immobilizing* activity exhibited was due to differences in fixability of the different complements Different native guinea pig sera might contain different subdetectable amounts of antitreponemal factors, the presence of which in analogy with the Neisser-Wechsberg phenomenon (*Neisser & Wechsberg* 1901, *Maaloe* 1946) *can be demonstrated* only upon the addition of small amounts of specific antibodies Another question arises whether the excess of hemolytic complement activity found by current qualitative methods is a criterion of excess of immobilizing activity It should be possible to resolve this question by making quantitative determinations of excess of immobilizing and hemolytic complement activity in the TPI test

But, even as long ago as 1909, *Browning & McKenzie* found that sera from different guinea-pigs with the same hemolytic complement activity

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STUDIES ON TOXIC PRODUCTS OF STAPHYLOCOCCUS[1]

By

KLAUS JENSEN, ERWIN NETER, EUGENE A. GORZYNSKI and HIROSHI ANZAI

Received 21.II.61

Although during past decades numerous studies have been carried out on the antigenic structure of staphylococcus and on the toxic products of this microorganism, many questions await elucidation. Recently *Jensen* isolated a toxic antigen (antigen A), which reacts with homologous antibodies occurring in all normal human sera and is toxic to the ileum of the guinea pig (1, 2, 3). This antigen was shown to be identical with the toxic principle in extract from staphylococcus described by *Dworetsky* and associates (4, 5, 6, 7). An extract from a coagulase positive strain (D) of *Staphylococcus aureus* was found to alter dermal reactivity of " [illegible] h is elicited also by endoto[illegible] in [illegible] or identical effect is produc[illegible] As shown by *Rantz* and associates (9, 10) and by *Neter & Gorzynski* (11, 12) many strains of staphylococci produce a heterogenetic antigen which is shared by numerous, but not all, species of gram positive bacteria. The present study was undertaken to obtain answers to the [illegible] igen A alter dermal reactivity [illegible] toxic extract, which alters der- [illegible] to the intestine of the guinea pig. (3) Does antigen A function as heterogenetic antigen of the Rantz type? The results of this investigation are embodied in this report.

MATERIALS AND METHODS

[illegible]

[1] [illegible] in part by research grant from National Institute of Allergy and Infectious Diseases, U.S. Public Health Service.

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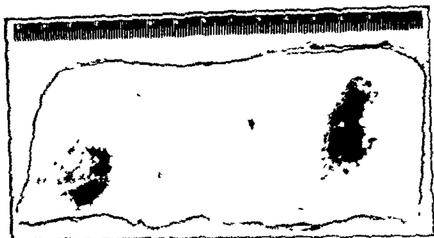


Fig. 1

Lesions at sites of intradermally injected epinephrine in rabbit injected intravenously with antigen A. Left: aqueous epinephrine. Upper right: epinephrine in oil. Lower right: saline.

norepinephrine. From these results it is evident that extract A alters the dermal reactivity of rabbits to epinephrine, similarly to the *previously studied* staphylococcal extract D and to viable staphylococci (8).

TABLE 1

Effect of Staphylococcal Extract A (Jensen) on Dermal Reactivity of Rabbits to Epinephrine

Reactions	Staphylococcal extract A (Jensen) µg/kg							
	100				20			
	Epinephrine 100 µg	Epinephrine in oil 200 µg	Norepinephrine (base) 100 µg	Saline	Epinephrine 100 µg	Epinephrine in oil 200 µg	Norepinephrine (base) 100 µg	Saline
	Number of rabbits							
Strong								
Hemorrhagic	3	4	0	0	1	1	0	0
Non hemorrhagic	1	3	0	0	0	0	0	0
Weak								
Hemorrhagic	0	0	0	0	0	0	0	0
Non hemorrhagic	4	2	0	0	2	1	1	0
Negative	4	3	3	9	1	2	2	3
Total number of rabbits	12	12	3	9	4	4	3	3

trifugation the clear supernatant was precipitated with HCl at pH 3 and centrifuged The precipitate which contained the antigen was dissolved in buffer solution and reprecipitated this time with *trichloracetic acid* *The precipitate was dissolved in* buffer solution and the insoluble fraction was discarded The antigen was then precipitated 3 times with 70 per cent alcohol and lyophilized This preparation consists mainly of polysaccharide Protein is absent

The staphylococcal extract D was prepared by extraction of a coagulase positive strain (phage type 52 42F 54) with trichloracetic acid according to method of *Boivin* It was clarified by filtration and precipitated with acetone or alcohol The precipitate was dialyzed against distilled water for 96 hours and the clarified residue in the bag dried from the frozen state The extract was obtained through the courtesy of Dr *C W Christensen* Difco Laboratories Detroit Michigan

Extract D and extract A were dissolved in phosphate buffer (pH 7 3) Stock solutions containing 1000 μg/ml were kept frozen until used

The method used for the determination of the toxic effect of these staphylococcal products on the ileum of the guinea pig was described previously Normal guinea pigs were killed with chloroform The ileum was removed immediately and segments each measuring 2 5 cm in length were placed into oxygenated Ringer solution (37° C) *The reactions were carried out in a 20 ml bath containing oxygenated Ringer* solution and the contractions were recorded directly on a kymograph Histamine as well as the antigens were added in a volume of 1 ml

For the epinephrine experiments the staphylococcal product was injected intravenously into female albino rabbits weighing between 1 5 to 2 5 kg Immediately thereafter epinephrine (aqueous or in oil) and physiologic saline solution (vol 0 1 ml) were injected into the shaved abdominal skin at different sites The resulting reactions were observed grossly at regular intervals

The hemagglutination test as described in detail by *Neter & Gorzynski* (11) was utilized for the demonstration of heterogenetic antigen in the extracts Briefly washed human red blood cells of blood group O (2 per cent) were treated with the toxic products in suitable dilution for 30 min in a water bath at 37° C The erythrocytes were then washed three times in phosphate buffer The suspension (vol 0 2 ml) was then added to staphylococcal antiserum in two fold serial dilutions The mixtures were incubated in a water bath for 30 min at 37° C and the resulting hemagglutination was read grossly after centrifugation at 1300 G for 3 min The *antiserum contained heterogenetic antibodies of the Rantz type in high titer and* was obtained from a rabbit injected with staphylococcus strain D

RESULTS

Effect of Antigen A on Dermal Reactivity of Rabbit to Epinephrine

Intravenous injection of extract A in amounts of 100 μg/kg followed *by intradermal injection* of 100 μg of aqueous epinephrine, 200 μg of epinephrine in oil, and of saline solution for control purposes, resulted in the appearance of a striking reaction at the sites of injected epinephrine Grossly the lesion is red, brownish or bluish (hemorrhagic) *Two such reactions are shown in Fig 1* No lesion whatever developed at the control site The results of titration experiments of extract A are summarized in Table 1

From the data shown in this table it is evident that of twelve rabbits *injected with extract A in amounts of 100* μg/kg eight showed lesions at the site of injected aqueous epinephrine and nine at that of epinephrine in oil Norepinephrine in amounts of 100 μg of base and saline solution failed to elicit any reaction It can be seen also that as little *as 20* μg/kg *of extract A caused the epinephrine reaction* in three out of four rabbits, and a weak reaction was seen in one rabbit injected with

Gel-precipitation Reactions between Antigen A and Extract D and Normal Human Serum

Figure 3 shows the gelprecipitation reaction between extract A and extract D and normal human serum as source of antibody The previously described normally occurring human antibody against antigen A gave a strong reaction with this antigen, but failed to give any reaction with extract D It is evident that extract D, also in this respect, is different from antigen A, and that extract D does not contain antigen A

Detection of Heterogenetic Antigen in Extract A

In order to ascertain whether extract A functions as heterogenetic antigen of the Rantz type, the following experiment was carried out Human erythrocytes were modified with extract A in concentrations of 1 5 to 100 μg/ml For control purposes, supernate of a *B subtilis* culture was used in serial dilutions in like manner The modified erythrocytes were then mixed with staphylococcal antiserum in serial dilutions The results of this experiment are presented in Table 2

TABLE 2
Staphylococcal Extract A (Jensen) and B Subtilis Hemagglutination by Staphylococcal (Rantz) Antiserum

Staphylococcal antiserum (Rantz) dilutions	Extract A (μg ml)				B subtilis			
	100	25	6	1.5	1 10	1 40	1 160	1 640
1 200	4	4	4	3	4	4	4	2
1 400	4	4	4	2	4	4	3	1
1 800	4	4	3	1	4	4	3	
1 1600	4	4	2		4	4	2	–
1 3200	4	4	1	–	4	3	1	–
1 6400	3	3	–		4	2	–	
1 12800	2	1			3	1		–
1 25600	1	1		–	2	1		
1 51200	1	–	–		1	–	–	–
1 102400			–	–	–	–	–	–
0		–	–	–	–	–	–	–

1 to 4 = Various degrees of hemagglutination — = No hemagglutination

Perusal of the table [illegible] or *B subtilis* we [illegible] dilution As litt [illegible] hemagglutinatio [illegible] antiserum It should be added that normal rabbit serum in dilutions of 1 100 or less proved to be ineffective

In order to determine the specificity of this hemagglutination reaction, the staphylococcal antiserum was absorbed with the sediment of a *B subtilis* culture and then tested in parallel with unabsorbed serum The results are presented in Table 3

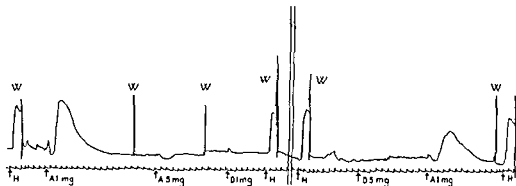


Fig 2

Kymogram showing the effect on isolated guinea pig ileum of extract A and extract D Time period – 10 sec, W – three washes with Ringers solution H – 1 3 μg histamine The double vertical lines indicate, that a new segment of ileum was used

Effect of Staphylococcal Extract D on the Ileum of the Guinea Pig

To ascertain whether extract D gives the same reaction on isolated guinea pig ileum as extract A, isolated segments of guinea pig ileum were challenged with both preparations Figure 2 shows the results of this experiment From the second part of the curve it is seen that extract D failed to elicit reactions even in a 5 mg dosage A subsequent challenge with 1 mg of antigen A gave the typical reaction of this antigen The first part of the curve shows the reactions on another segment of ileum Antigen A gives the typical reaction, resulting in a refractory state to a subsequent challenge with the identical antigen No reactions were elicited by extract D in this segment of ileum It is concluded, therefore, that antigen D does not produce the same toxic effect on isolated guinea pig ileum as does extract A

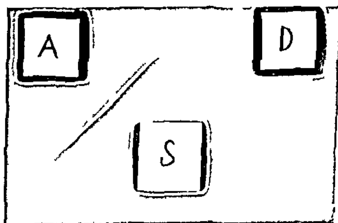


Fig 3

Gel precipitation reaction between normal human serum (S) extract A (A) and extract D (D)

Gel precipitation Reactions between Antigen A and Extract D and Normal Human Serum

Figure 3 shows the gelprecipitation reaction between extract A and extract D and normal human serum as source of antibody The previously described normally occurring human antibody against antigen A gave a strong reaction with this antigen, but failed to give any reaction with extract D It is evident that extract D, also in this respect, is different from antigen A, and that extract D does not contain antigen A

Detection of Heterogenetic Antigen in Extract A

In order to ascertain whether extract A functions as heterogenetic antigen of the Rantz type, the following experiment was carried out Human erythrocytes were modified with extract A in concentrations of 1 5 to 100 μg ml For control purposes, supernate of a *B subtilis* culture was used in serial dilutions in like manner The modified erythrocytes were then mixed with staphylococcal antiserum in serial dilutions The results of this experiment are presented in Table 2

TABLE 2

Staphylococcal Extract A (Jensen) and B Subtilis Hemagglutination by Staphylococcal (Rant) Antiserum

Staphylococcal antiserum (RaC) Dilutions	Extract A (μg ml)				B s M I s			
	100	25	6	1.5	1 10	1 40	1 160	1 640
1 200	4	4	4	3	4	4	4	2
1 400	4	4	4	2	4	4	3	1
1 800	4	4	3	1	4	4	3	–
1 1600	4	4	2	–	4	4	2	–
1 3200	4	4	1		4	3	1	
1 6400	3	3			4	2	–	–
1 12800	2	1	–		3	1		–
1 25600	1	1			2	1	–	–
1 51200	1		–		1	–		–
1 102400		–	–	–	–		–	–
0	–	–	–	–		–	–	–

1 to 4 = Various degrees of hemagglutination — = No hemagglutination

Perusal of the table reveals that erythrocytes treate[illegible]

[illegible] antiserum It should be added that normal rabbit serum in dilutions of 1 100 or less proved to be ineffective

In order to determine the specificity of this hemagglutination reaction, the staphylococcal antiserum was absorbed with the sediment of a *B subtilis* culture and then tested in parallel with unabsorbed serum The results are presented in Table 3

TABLE 3

Agglutination by Staphylococcal (Rantz) Antiserum of Erythrocytes Modified by Staphylococcal Extract A (Jensen) and by Supernate from B Subtilis

Staphylococcal antiserum (151C) dilutions	Erythrocytes modified with extract A		Erythrocytes modified with *B subtilis* supernate	
	Antiserum unabsorbed	Antiserum *absorbed* with *B subtilis*	Antiserum unabsorbed	Antiserum *absorbed* with *B subtilis*
1 20	4	–	4	–
1 40	4		4	–
1 80	4		4	–
1 160	4	–	4	–
1 320	4	–	4	–
1 640	4		4	
1 1280	4		3	–
1 2560	3		3	–
1 5120	3		2	
1 10240	2	–	1	–
1 20480	1	–	1	–
1 40960	–	–	–	–

1 to 4 = *Various degrees of hemagglutination* – = *No hemagglutination*

It is evident from the data shown in this table that *B subtilis* removes the antibodies causing hemagglutination of erythrocytes modified by extract A It is concluded, therefore, that extract A contains heterogenetic antigen of the Rantz type It should be added that staphylococcal extract D, which alters dermal reactivity of the rabbit to epinephrine, does not modify erythrocytes for agglutination by the identical antiserum, and thus differs strikingly from extract A

With these results at hand, it was of interest to determine whether intravenous injection of extract A engenders heterogenetic antibodies It was found that a single intravenous injection of extract A (100 µg) into three rabbits resulted in the appearance of heterogenetic antibody of the Rantz type in titer of 1 1280, the preimmunization serum specimens failed to cause hemagglutination even in a dilution of 1 10 The antibodies are removed by absorption with *B subtilis*

Effect of Heterogenetic Antibodies on Toxicity of Antigen A

In order to determine whether the heterogenetic antibody protects rabbits against the epinephrine reaction, described above, rabbits were injected with extract A and challenged with epinephrine at intervals of 2 weeks It was found that the epinephrine reaction could be elicited in rabbits whose sera contained heterogenetic antibodies either in titer of less than 1 10 or of 1 1280 In addition neutralization tests with two staphylococcal antisera were undertaken Antiserum 154C obtained by the immunization with strain D, contained heterogenetic antibodies in high titer and antiserum 170C, procured by injection of the Smith

TABLE 4

Effect of Staphylococcal Antiserum on Toxicity of Staphylococcal Extract A (Jensen) in Rabbits

Extract A (Jensen) with or without staphylococcal antiserum	Epinephrine 100 µg		Epinephrine in oil 200 µg		Saline	
	Reactions					
	Positive	Negative	Positive	Negative	Positive	Negative
	Number of rabbits					
Antigen (100 µg/kg)	8	4	9	3	0	9
(20 µg/kg)	3	1	2	2	0	3
Antigen (100 µg/kg) + antiserum (154C) (1/40) 1 ml/kg	3	1	3	1	0	4
Antigen (100 µg/kg) + antiserum (170C) (1/40) 1 ml/kg	2	0	2	0	0	2

strain, did not. The latter antiserum, however, contained protective antibodies, as measured in the mouse protection test by Dr. M. W. Fisher of Parke, Davis and Company, Detroit, Michigan. The results of the experiment are recorded in Table 4 and reveal that the heterogenetic antibody of the Rantz type does not abolish alteration in reactivity to epinephrine elicited by extract A.

Separation between Antigen A and Heterogenetic Antigen of the Rantz Type

In the foregoing it has been shown that extract A contains heterogenetic antigen of the Rantz type. However, it has not been determined whether heterogenetic antigen is identical with antigen A, or if the two antigens are different components of the same extract. In previous communications (2, 3, 10) it has been shown that the antibody against antigen A occurs in high titers in all normal human sera, whereas the heterogenetic antibody of the Rantz type only occurs in some, but not all, normal human sera. By means of the gel precipitation technique it has not been possible to demonstrate antigen A in microorganisms other than pathogenic staphylococci, such as *B. subtilis* used in these experiments. To prove whether antigen A and the heterogenetic antigen of the Rantz type are different molecules in extract A the following experiments were carried out.

Rabbit staphylococcal antiserum which gave positive hemagglutination reactions with both erythrocytes coated with extract A and erythrocytes coated with supernate from *B. subtilis* culture, was absorbed with *B. subtilis*, and both reactions became negative (Table 3). By means of the gel precipitation reaction (Fig. 4) between extract A and the absorbed and unabsorbed rabbit sera and normal human serum it

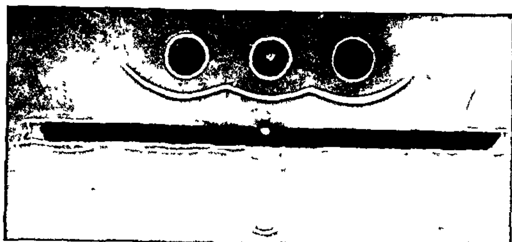


Fig 4
Gel-precipitation reaction between extract A (in the long well), rabbit antiserum (*left cup*), *rabbit antiserum absorbed with Rantz antigen* (middle cup), and normal human serum (right cup)

was shown that all three sera contained antibody against antigen A in large amounts, and that this reaction was not abolished by previous absorption of the heterogenetic antibody It is concluded then that heterogenetic antigen and antigen A are not identical

This conclusion is supported also by the results of the following experiment By means of the hemagglutination test with erythrocytes coated with supernate of a *B subtilis* culture, ten normal human sera were selected which did not contain heterogenetic antibody The gel-precipitation test with extract A revealed that all these sera contained antibodies against antigen A

DISCUSSION

The present study has revealed that an extract containing antigen A is toxic to the ileum of the guinea pig, alters the dermal reactivity of the rabbit to epinephrine, and reacts with homologous antibodies as well as heterogenetic antibodies of the Rantz type In contrast, the toxic extract D only alters the epinephrine reactivity For these reasons, the question arises as to whether extract A is a single substance with multiple activities or whether, in fact, it is a mixture of several components The experiments reported here have shown that extract A contains at least three different components

1 Antigen A, characterized by its ability to react with the homologous antibody occurring in all normal human sera and by its toxic effect on isolated guinea pig ileum

2 Toxin D, characterized by its ability to alter the dermal reactivity of the rabbit to epinephrine

3 Heterogenetic antigen of the Rantz type, characterized by its

ability to coat erythrocytes and make them agglutinable by heterogenetic antibodies of the Rantz type

It has been postulated by *Oeding* (14) that antigen A is a mixture of different antigens, and that the gel-precipitation reaction between antigen A and normal human sera is the result of several different antigen-antibody systems The experiments reported here not only show that the extract containing antigen A is a mixture of at least three different components, but also support the previous data (3) demonstrating that the agglutination of staphylococci in normal human sera, the gel-precipitation reaction between normal human serum and extract A, and the toxic effect of extract A on isolated guinea pig ileum are all due to the same antigen This antigen is referred to as antigen A

As yet, the chemical composition of extract D is unknown In particular, it will be of interest to determine whether this extract contains a lipopolysaccharide moiety, for such lipopolysaccharide complexes of gram-negative bacteria also alter the reactivity of the rabbit to epinephrine In addition, it has been shown recently that the polysaccharide-free lipoid A component of the *E coli* lipopolysaccharide complex is also active (13) It is well known that antibodies directed against the specific polysaccharide of the endotoxin (lipopolysaccharide) complex of gram negative bacteria do not abolish numerous biologic activities, such as fever, toxicity, production of the local and generalized Schwartzman reactions In this connection, it may be pointed out that two staphylococcal antisera failed to prevent the epinephrine reaction elicited by extract A

Future efforts must be directed toward further characterization of the toxic extracts, and particularly toward separation of various molecules with different biologic activities of extract A

Finally, the fact that two staphylococcal extracts, prepared in different laboratories, alter dermal reactivity of rabbits to epinephrine strongly suggests that this activity is not due to contamination with endotoxin from gram negative bacteria

SUMMARY

A comparative study of staphylococcal extract A and of extract D yielded the following results (1) Extract A is toxic to the intestine of the guinea pig, strikingly alters dermal reactivity of the rabbit to epinephrine, and reacts with heterogenetic antibodies of the Rantz type The latter conclusion is based on hemagglutination and hemagglutinin absorption tests (2) The staphylococcal extract D, like extract A, alters dermal reactivity of the rabbit to epinephrine, but, in contrast to extract A does not produce toxic effects on the guinea pig intestine and fails to react with heterogenetic antibodies of the Rantz type (3) Heterogenetic antibodies of the Rantz type fail to inhibit the epinephrine lesion elicited by extract A (4) The data obtained in this investigation

suggest that extract A represents a mixture of three or more components with different biologic activities (a) Antigen A characterized by its ability to react with the homologous antibody occurring in all normal human sera and by its toxic effect on isolated guinea pig ileum (b) A component, like extract D, characterized by its ability to alter the dermal reactivity of the rabbit to epinephrine (c) Heterogenetic antigen of the Rantz type, characterized by its ability to coat erythrocytes and make them agglutinable by heterogenetic antibodies

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14 *Oeding P* Antigenic properties of staphylococcus aureus Bacteriol Reviews *24* 374 1960

AUS STATENS SERUMINSTITUT (INTERNATIONALE SALMONELLA UND ESCHERICHIA CENTRALE) KOPENHAGEN L'INSTITUT NATIONAL DE RECHERCHES VÉTÉRINAIRES UCCLE (DIREKTOR DR R WILLEMS) UND L'INSTITUT D'HYGIÈNE ET D'ÉPIDÉMIOLOGIE BRUXELLES (DIREKTOR DR A LAFONTAINE)

EINE NEUE SALMONELLA SPECIES MIT EINEM NEUEN O ANTIGEN SALMONELLA UCCLE = 54 g s t –

Von

F KAUFFMANN J THOMAS und F VAN OYE

Eingegangen 4 1 1961

Eine neue *Salmonella species* wurde in Uccle (Belgien) aus importierten tierischen Futtermehlen isoliert und mit *Salmonella uccle* bezeichnet

Die neue *Salmonella species* verhielt sich in der biochemischen Un

Lactose Sac

von Urea und

bildung und negative Reaktion in *Stern* s Glycerinfuchsin Bouillon Prompte Spaltung von Arabinose Dulcit Glukose (mit Gas) Maltose Mannit (mit Gas) Rhamnose Sorbit Trehalose und Xylose Positive Reaktion in flussigen Ammonium Media mit Glukose und Natrium Citrat Nitrat wurde reduziert die *Voges Proskauer* Reaktion war negativ und die Methylrot Reaktion positiv Der KCN Test war negativ In d tartrat l tartrat Natrium Citrat und Mukat war die Reaktion positiv *nach 1 Tage* I tartrat ergab nach 14 Tagen und Natrium Malonat nach 4 Tagen *eine negative* Reaktion

Die serologische Untersuchung ergab dass die monophasische Kultur die H Antigene g s t *enthalt* und zu einer neuen O Gruppe 54 geh

Obwohl S

reagie

nicht … zur Herstellung eines reinen O 42 Serums sollte daher dieses Serum mit *S uccle absorbiert* werden … r be-

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… die serologische Verschiedenheit dieser O Gruppen

ZUSAMMENFASSUNG

Es wird eine neue *Salmonella species mit einem neuen O Antigen Salmonella uccle* = 54 g s t – aus importierten tierischen Futtermitteln isoliert beschrieben

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variants with age *Hamre* (1949) observed no significant difference in the mortality of 6 hour and 3 day-old cultures of K pneumoniae Seven-day-old E coli are killed more rapidly by ultrasonic treatment than one-day old cultures (*Horwood et al* 1950) Working with 15 and 48 hour-old E coli, the same ultrasonic killing effect was obtained by Grün & Steller (1955) The dispersing effect of low intensity (0 56 W/cm²) ultrasonic waves (800 kC) on BCG cultures was faster with 5-week than with 3 day cultures, but the cultures were equally sensitive to the bactericidal effect of the oscillations (*Mackeprang* 1960) Yeast cells were more susceptible to ultrasonic waves in the budding and actively growing state than at rest (*Beckwith & Weaver* 1936, *Kinsloe et al* 1954) Large rods were easier disintegrated than small rods, and rod shaped bacteria are generally assumed to be more susceptible than cocci (*Beard & Gantvoort* 1938), although this connection is not very close (*Steller & Grün* 1956) *Hesselberg* (1958) found that young cultures of E coli were more resistant to ultrasonic treatment than older cultures (18 hours > 3 days > 5 days) *Seki et al* (1958) reported that 12-hour cultures of Clostridium tetani were more easily disintegrated by Mickle (1948) or sonic treatment than those grown for 24 hours Frog eggs were most sensitive to ultrasonic treatment in the first division stage (*Chambers & Harvey* 1931) Cells of the skin are most easily destroyed when they are rapidly dividing (cf *Grabar* 1953)

During the lag and logarithmic stages of growth, the bacteria are subjected to great morphological and biochemical alterations (*Malmgren & Hedén* 1947) The susceptibility of the cells to various deleterious chemical and physical agents is also profoundly altered (*Dubos* 1948) If also a different resistance to disintegration during the various growth stages could be shown, it would not only give information on the physiological state of the bacteria but also be of importance in practical disintegration work[1]

Proteus was chosen as the test organism, because the volume variations under the different growth stages of Proteus are particularly wide (*Malmgren & Hedén* 1947) Three phases of Proteus, A, B and C (*Belyavin* 1951) are distinguishable by cellular and colonial morphology Phase A (the usual form of freshly isolated strains) is characterized by cells which have a uniform bacillary morphology and swarm intermittently on nutrient agar Phase B cells are usually nonmotile, and the morphology is pleomorphic The bulk of the population, however, consists of shorter and plumper rods than phase A Phase C cells are usually long rods or filaments which swarm giving a continuous film on nutrient agar It was expected that due to the different morphology of the phases sonic waves might give effects which could elucidate the mechanism of sonic disintegration of bacteria The morphological

[1] A preliminary report on this work has been presented earlier (*Edebo & Hedén* 1958)

DEPARTMENT OF BACTERIOLOGY, KAROLINSKA INSTITUTET STOCKHOLM 60 SWEDEN (HEAD PROFESSOR B MALMGREN)

THE SENSITIVITY OF PROTEUS MIRABILIS TO SONIC OSCILLATION IN VARIOUS PHASES AND GROWTH-STAGES[1]

By

LARS LDEBO

Received 21 iii 61

For the purpose of disintegrating cells, sonic and ultrasonic oscillation has been used for the last three decades and several investigations have been devoted to it (cf *Bergmann* 1949, *der Ultraschall in der Medizin*, 1949, 1950, *Naimark et al* 1951, *Grabar* 1953, *Kelly* 1957, *Hesselberg* 1958, *Mackeprang* 1960) The action of sonic waves is manifold, but three main properties should be mentioned heating, oxidizing and morphology destructing effects Only the destructive effects, are desired, when cells are to be disintegrated They have long been known to be intimately connected with cavitation (*Johnson* 1929, *Schmitt & Uhlemeyer* 1930) *Cavitation is usually described as the formation and* disappearance of "holes", or cavities in the liquid under treatment The importance of dissolved gases for the production of cavitation in ordinary types of sonic equipment, and the importance of cavitation in producing disintegration plus several other sonic effects, was stressed *in a series of experiments by Grabar and collaborators (cf Grabar 1953)*

The sensitivity of several different kinds of cells to sonic disintegration has been extensively studied However, earlier observations on the disintegration sensitivity of micro organisms during various stages of growth are mentioned mostly incidentally in connection with other observations *Williams & Gaines (1930) obtained identical killing effects* by sonic oscillation of a 13-hour and a 27-hour culture of E coli *Chambers & Gaines* (1932) found that young E coli cells were more rapidly killed than old cells, the sensitivity decreased with increased age (12 hour > 5 day > 8 day > 14 day cultures) These authors suggested that the *increased resistance was due to a selection of resistant*

This work was made possible by a grant from *the Wallenberg Foundation* The *technical assistance provided by Miss Ella Spet and Mr Yngve Andersson* is gratefully acknowledged

[1] In this article the term phases is reserved for Proteus phase variation (*Belyavin* 1951) while stages stands for the different developmental stages in the growth cycle

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[1] A preliminary report on this work has been presented earlier (*Edebo & Heden* 1958)

data themselves were also thought to be of interest, since no quantitative comparison of the different Belyavin phases so far seems to have been published

MATERIALS AND METHODS

All experiments were performed with spontaneous variants of the same original strain of Proteus mirabilis (collected from a urinary specimen) The experiments are numbered with arabic numerals (1–5) and equally so are the tables figures and photomicrographs illustrating the same experiments The experiments 1 and 2 were performed with Proteus in phase A, experiment 3 in phase B and experiment 5 in phase C The bacteria investigated in experiment 4 were not entirely in phase C but showed very faint concentric swarming which is a characteristic of phase A *Coetzee* (1960) has demonstrated the presence of further Proteus phases and still more variants have been observed (*Edebo* unpublished results)

A Raytheon magnetostrictive oscillator (model DF 101, 10 kC, 250 watt) was used, always at the highest output effect at resonance frequency The treatment assembly was continuously cooled and the temperature increase in the material never exceeded 2° C, the temperature at the start of the treatment never exceeded +15° C Originally the resonance frequency was adjusted with the aid of a gramophone pick-up which gave an oscillograph reading in turn calibrated by means of a tone generator This calibration could be omitted as experience with the equipment accumulated

EXPERIMENTAL

The growth from seven Difco agar plates incubated for eighteen hours at 37° C was harvested in 35 ml sterile saline (0 90 per cent) The suspension was transferred to a 6 litre Erlenmeyer flask containing 3 litres of preheated (37° C) Difco broth (resulting cell count. $3-6 \times 10^8$ bact./ml) The flask was placed on a rotary shaker table (120 r p m) in a 37° C incubator room Specimens, 200 ml each were taken from the broth culture immediately after inoculation and at various times during growth, immediately cooled in an ice water bath, and then centrifuged for half an hour at $3{,}000 \times g$ in a refrigerated centrifuge The supernatants were discarded and preparations for photomicrography were prepared from the sediment by methods similar to those of *Malmgren & Hedén* (1947) The cover slip was pressed down gently for about a minute, and a field was always selected where one or two bacteria were still capable of moving freely The bacteria were photographed in a Zeiss Photomikroskop The photomicrographs were enlarged to about 4,000 times, and the length and diameter of 200 cells were measured by a transparent ruler, or even more simply by using a plexiglass plate with two scales set at right angles The volume and the area of the bacteria were calculated according to the formulae (*Malmgren & Hedén* 1947)

$$V = \frac{\pi d^2}{4}\left(l - \frac{d}{3}\right)$$

$$T = 2\pi d l$$

(V = volume, T = area, l = length, d = diameter)

An expression of the shape was obtained through the quotient (Q) between length and diameter ($Q = \frac{l}{d}$).

The standard deviation, σ, and the standard error of the mean ε (M) were calculated in the usual way by the use of an arbitrary origin, (M₀) (*Dahlberg* 1948).

After centrifugation the bacterial sediment was suspended in 12 ml of 0.9 per cent saline. One ml was mixed with 9 ml saline containing 0.5 per cent formaldehyde, and after suitable dilution, at least 500 cells were counted in a Buerker chamber (depth 0.02 or 0.01 mm). The remaining 11 ml were treated in the sonic oscillator for seven minutes and then counted as just outlined. The disintegration effect is expressed as the difference between the logarithms of the initial number and the cell number after sonic treatment. In three experiments (2, 4 and 5) the growth curves were determined with the aid of the mentioned counting method. Since it is very difficult to determine concentrations below 2×10^7 bact./ml in a bacterial counting chamber with the depth of 0.01 mm, big inocula were chosen. This amount of material made it possible to use longer periods of sonic treatment. It caused a large reduction in the number of bacteria per ml, and reduced the relative counting error.

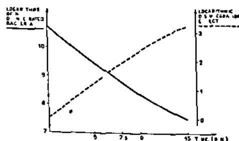


Fig. 4.
Effect of time of sonic treatment on disintegration of Proteus phase A.

When frequency and the volume in the cup were standardized it was observed that the logarithmic disintegration plotted against time was almost a straight line, which only bent a little over lengthened periods (Fig. 4). This linear relationship made the difference between the logarithms of the number of untreated and sonic treated cells the best tool for estimating the disintegration effect since it was not influenced by the initial concentration of bacteria within the range of concentrations used.

When the volume (V), area (T), length diameter quotient (Q), and logarithmic disintegration effect (D) calculated for regular intervals after inoculation (Table 1–5) are plotted against time (Fig. 1–5) it is observed that the size and shape, as well as the susceptibility to sonic

TABLE 1

Disintegration of Phase A Cells

Growth time (hours)	Log disintegration	Volume (μ^3)			Area (μ^2)			Quotient 1 d
		Mean	σ	ε (M)	Mean	σ	ε (M)	
0	1 74	0 216	0 079	0 0056	1 91	0 51	0 036	2 24
1	2 31	0 368	0 169	0 0119	2 79	0 89	0 063	2 70
1½	2 51	0 458	0 159	0 0112	3 31	0 82	0 058	2 78
2	2 89	0 718	0 217	0 0153	4 53	1 01	0 071	2 93
2½	2 93	*0 637*	*0 191*	*0 0135*	*4 28*	*0 91*	*0 064*	*3 02*
4	2 67	0 482	0 152	0 0108	3 31	0 78	0 054	2 50
24	1 42	0 276	0 095	0 0067	2 14	0 55	0 038	1 71

Before sonic treatment 7 8 × 10^9 – 9 6 × 10^{10} bact/ml
After sonic treatment 1 3 × 10^7 – 3 7 × 10^9 bact/ml

TABLE 2

Disintegration of Phase A Cells

Growth time (hours)	Growth (Bact/ml × 10^{-8})	Log disint	Volume (μ^3)			Area (μ^2)			Quotient 1 d
			Mean	σ	ε (M)	Mean	σ	ε (M)	
0	6 4	1 92	0 272	0 090	0 0063	2 19	0 56	0 040	2 36
1	*7 4*	*1 97*	*0 568*	*0 186*	*0 0131*	*3 65*	*0 95*	*0 060*	*2 16*
1½	7 9	2 11	0 795	0 286	0 0202	4 67	1 19	0 084	2 35
2	10 2	2 56	0 782	0 295	0 0209	4 86	1 32	0 093	2 97
2½	12 4	2 69	0 812	0 241	0 0170	5 00	1 08	0 077	3 05
4	26 1	2 84	0 569	0 176	0 0124	3 72	0 79	0 056	2 41
24	57 7	*1 83*	*0 313*	0 105	*0 0074*	*2 36*	0 56	*0 040*	1 85

Before sonic treatment 9 6 × 10^9 – 1 2 × 10^{11} bact/ml
After sonic treatment 3 4 × 10 – 1 8 × 10^9 bact/ml

TABLE 3

Disintegration of Phase B Cells

Growth time (hours)	Log disintegration	Volume (μ^3)			Area (μ^2)			Quotient 1 d
		Mean	σ	ε (M)	Mean	σ	ε (M)	
0	1 84	0 249	0 097	0 0068	2 00	0 58	0 041	2 01
1	2 21	0 354	0 132	0 0093	2 70	0 72	0 051	2 45
1½	2 46	0 390	0 149	0 0105	2 89	0 82	0 058	2 45
2	2 61	0 458	0 156	0 0111	3 21	0 80	0 056	2 40
2½	2 50	0 336	0 123	0 0087	2 55	0 69	0 049	2 17
4	2 23	0 226	0 079	0 0056	1 92	0 50	0 035	1 96
24	(1 95)	0 216	0 075	0 0053	1 83	0 47	0 033	1 76

Before sonic treatment 9 9 × 10^9 – 1 1 × 10^{11} bact/ml
After sonic treatment 4 4 × 10 – 1 3 × 10^9 bact/ml

TABLE 4

Disintegration of Phase C (A) Cells

Growth time (hours)	Growth (Bact ml $\times 10^{-8}$)	Log disint	Volume (μ^3)			Area (μ^2)			Quotient $\frac{1}{d}$
			Mean	σ	ε (M)	Mean	σ	ε (M)	
0	5.5	2.32	0.328	0.161	0.0115	2.63	0.88	0.062	2.79
1	6.3	2.75	0.578	0.228	0.0161	4.00	1.13	0.080	3.20
1½	10.4	2.99	0.585	0.277	0.0196	4.07	1.31	0.092	3.46
2	11.6	3.58	0.458	0.173	0.0122	3.73	1.08	0.077	4.37
2½	21.4	3.42	0.455	0.156	0.0110	3.57	0.92	0.065	3.82
3	16.6	3.70	0.359	0.142	0.0101	2.99	0.84	0.059	3.58
4	32.3	3.25	0.283	0.138	0.0097	2.43	0.88	0.062	2.92
5	48.7	3.39	0.238	0.108	0.0076	2.10	0.75	0.053	2.57
24	84.9	1.83	0.192	0.094	0.0067	1.71	0.59	0.041	1.98

Before sonic treatment $7.9 \times 10^{9} - 3.5 \times 10^{10}$ bact./ml
After sonic treatment $9.3 \times 10^{8} - 5.1 \times 10^{9}$ bact./ml

TABLE 5

Disintegration of Phase C Cells

Growth time (hours)	Growth (Bact ml $\times 10^{-8}$)	Log disint	Volume (μ^3)			Area (μ^2)			Quotient $\frac{1}{d}$
			Mean	σ	ε (M)	Mean	σ	ε (M)	
0	2.7	2.49	0.505	0.225	0.0159	3.55	1.31	0.093	2.80
1	3.2	2.17	0.749	0.318	0.0225	4.68	1.46	0.103	2.99
1½	3.7	2.52	1.060	0.417	0.0295	6.24	1.93	0.136	3.73
2	6.2	2.77	0.948	0.365	0.0258	6.09	1.79	0.127	4.42
2½	6.3	2.84	0.730	0.290	0.0205	5.06	1.64	0.116	4.32
3	7.8	2.57	0.911	0.405	0.0286	5.79	2.17	0.153	4.10
4	10.5	2.65	0.570	0.215	0.0152	3.89	1.20	0.085	2.95
5	16.2	2.59	0.585	0.281	0.0199	3.87	1.54	0.109	2.65
24	60.2	2.17	0.286	0.100	0.0070	2.32	0.59	0.041	2.29

Before sonic treatment $6.6 \times 10^{9} - 4.9 \times 10^{10}$ bact./ml
After sonic treatment $2.1 \times 10^{7} - 3.3 \times 10^{8}$ bact./ml

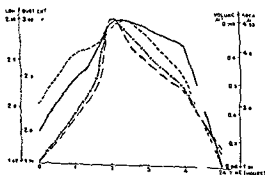


Fig. 1
Disintegration of Proteus phase A

Log disintegration —— Volume - - - Area -+-+- Quotient $\frac{1}{d}$ ---

TABLE 1

Disintegration of Phase A Cells

Growth time (hours)	Log disintegration	Volume (μ^3) Mean	σ	ε (M)	Area (μ^2) Mean	σ	ε (M)	Quotient l/d
0	1.74	0.216	0.079	0.0056	1.91	0.51	0.036	2.24
1	2.31	0.368	0.169	0.0119	2.79	0.89	0.063	2.70
1½	2.51	0.458	0.159	0.0112	3.31	0.82	0.058	2.78
2	2.89	0.718	0.217	0.0153	4.53	1.01	0.071	2.93
2½	2.93	0.637	0.191	0.0135	4.28	0.91	0.064	3.02
4	2.67	0.482	0.152	0.0108	3.31	0.78	0.054	2.50
24	1.42	0.276	0.095	0.0067	2.14	0.55	0.038	1.71

Before sonic treatment $7.8 \times 10^9 - 9.6 \times 10^{10}$ bact./ml
After sonic treatment $1.3 \times 10 - 3.7 \times 10^9$ bact./ml

TABLE 2

Disintegration of Phase A Cells

Growth time (hours)	Growth (Bact/ml $\times 10^{-8}$)	Log disint	Volume (μ^3) Mean	σ	ε (M)	Area (μ^2) Mean	σ	ε (M)	Quotient l/d
0	6.4	1.92	0.272	0.090	0.0063	2.19	0.56	0.040	2.36
1	7.4	1.97	0.568	0.186	0.0131	3.05	0.85	0.060	2.16
1½	7.9	2.11	0.795	0.286	0.0202	4.67	1.19	0.084	2.35
2	10.2	2.56	0.782	0.295	0.0209	4.86	1.32	0.093	2.97
2½	12.4	2.09	0.812	0.241	0.0170	5.00	1.08	0.077	3.05
4	26.1	2.84	0.569	0.176	0.0124	3.72	0.79	0.056	2.41
24	57.7	1.83	0.313	0.105	0.0074	2.36	0.56	0.040	1.85

Before sonic treatment $9.6 \times 10^9 - 1.2 \times 10^{11}$ bact./ml
After sonic treatment $3.4 \times 10^7 - 1.8 \times 10^9$ bact./ml

TABLE 3

Disintegration of Phase B Cells

Growth time (hours)	Log disintegration	Volume (μ^3) Mean	σ	ε (M)	Area (μ^2) Mean	σ	ε (M)	Quotient l/d
0	1.84	0.239	0.097	0.0068	2.00	0.58	0.041	2.01
1	2.21	0.354	0.132	0.0093	2.70	0.72	0.051	2.45
1½	2.40	0.390	0.149	0.0105	2.89	0.82	0.058	2.45
2	2.61	0.458	0.156	0.0111	3.21	0.80	0.056	2.40
2½	2.50	0.336	0.123	0.0087	2.55	0.69	0.049	2.17
4	2.23	0.226	0.079	0.0056	1.92	0.50	0.035	1.96
24	(1.95)	0.216	0.075	0.0053	1.83	0.47	0.033	1.76

Before sonic treatment $9.9 \times 10^9 - 1.1 \times 10^{11}$ bact./ml
After sonic treatment $4.4 \times 10 - 1.3 \times 10^9$ bact./ml

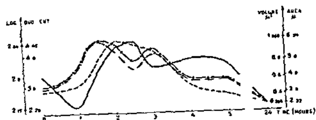


Fig 5
Disintegration of Proteus phase C
Log disintegration —— Volume - - Area -+-+- Quotient $\frac{1}{d}$ ---

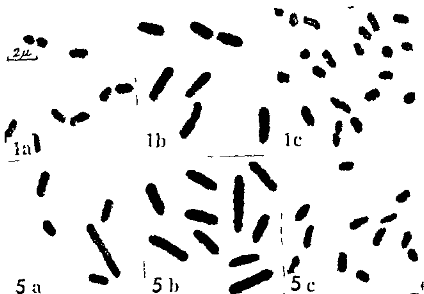


Fig 6

Photo 1 Proteus mirabilis phase A
a After 18 hours growth on nutrient agar, immediately after inoculation in broth
b After 2 hours' growth in broth
c After 24 hours' growth in broth

Photo 5 Proteus mirabilis phase C
a After 18 hours' growth on nutrient agar, immediately after inoculation in broth
b After 1½ hours' growth in broth
c After 24 hours' growth in broth

disintegration, were increased during the late lag and the logarithmic stages of growth In order to simplify the comparison of different values for volume, area, quotient and disintegration effect at different growth stages within the same experiment, the maximum value of each parameter are given the same ordinate in the diagrams, and so are the

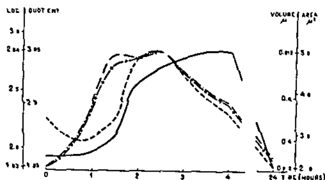


Fig 2
Disintegration of Proteus phase A
Log disintegration —— Volume - - - Area -+-+- Quotient $\frac{1}{d}$ ---

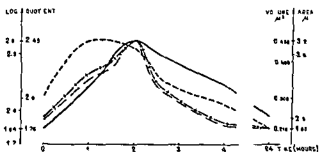


Fig 3
Disintegration of Proteus phase B
Log disintegration —— Volume - - Area -+ +- Quotient $\frac{1}{d}$ ---

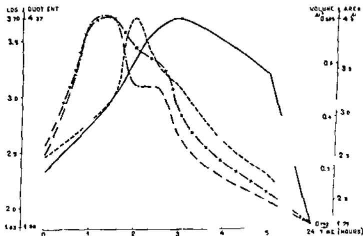


Fig 4
Disintegration of Proteus phase C (A)
Log disintegration — Volume - Area -+ + Quotient $\frac{1}{d}$ --

minimum values The scale of the logarithmic disintegration effect is the same in all figures and the other scales are adjusted to it In this fashion it is clearly demonstrated in the figures that the correlation between volume, area, quotient and disintegration effect was not very close The disintegration effect was still considerably increased when the size of the bacteria returned to the original value Table 6 is a comparison between the different experiments (1–5) There it is demonstrated that the relationship between disintegration effect and cell morphology varied considerably between the different Proteus phases This is most pronounced in experiment 5 where the changes of volume, area and quotient were greater than in all the other experiments, but the changes in disintegration effect were smallest This is visualized in the factors (f_V, f_T, f_Q) which are the quotients of the difference between maximum and minimum values of the logarithmic disintegration effect and the volume, area and quotient respectively

$$f_V = \frac{D_{max} - D_{min}}{V_{max} - V_{min}} \qquad f_T = \frac{D_{max} - D_{min}}{T_{max} - T_{min}} \qquad f_Q = \frac{D_{max} - D_{min}}{Q_{max} - Q_{min}}$$

All the factors are lower in experiment 5 than in any other run The different points of the diagrams are obtained by multiplying the difference between the actual value and the minimum of its parameter with the factor, thus obtaining the distance from the minimum along the ordinate D_{max} is the maximum value of the logarithmic disintegration in the actual experiment and D_{min} the minimum value The symbols V, T and Q are, similarly, volume, area and quotient, respectively

When experiment 4 was run, a new bacterial counting chamber was used Later it was discovered that its cover slip loosened during the counting performance which might account for the unusually scattered disintegration values The disintegration curve in Fig 4 is plotted between these values which are drawn as crosses

DISCUSSION

Strong evidence (*Rotman* 1956, *Edebo* 1961) has been presented that, from the biological point-of view, the mechanism of sonic disintegration of bacteria is a two step process The first involves structural damage of the cell wall, while the second step includes dispersion of the proto-
plasm [illegible]
go gr [illegible]

Th [illegible]
ism f [illegible]
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large [illegible] during the lag and the logarithmic stages of growth In their report it was not mentioned to which phase their strain

TABLE 6

Comparison of Disintegration Effect with Volume, Area and $\frac{l}{d}$ Quotient

Experimental nr	Log disintegration			Volume (μ^3)			Area (μ^2)			Quotient $\left(\frac{l}{d}\right)$		
	Max	Min	f	Max	Min	f_V	Max	Min	f_T	Max	Min	f_Q
1	2.93	1.42	Definition factor = 1	0.718	0.216	3.01	4.53	1.91	0.58	3.02	1.71	1.15
2	2.84	1.83		0.812	0.272	1.87	5.00	2.19	0.36	3.05	1.85	0.84
3	2.61	1.84		0.458	0.216	3.18	3.21	1.83	0.56	2.45	1.76	1.12
4	3.70	1.83		0.585	0.192	4.76	4.07	1.71	0.79	4.37	1.98	0.78
5	2.84	2.17		1.060	0.286	0.87	6.24	2.32	0.17	4.42	2.29	0.32

(*Briggs* 1947 *Jacobs & Thornley* 1954 *Hesselberg* 1958) This small decrease does not influence our results measurably however

If a relatively small rupture of the cell wall is all that is needed to disperse the bacterial protoplasm (*Marr & Cota Robles* 1957) a cell with a large surface could be expected to be more easily disintegrated than a small cell This may be due either to a greater risk for a hit or to the larger total force exerted on a large cell The pressure (per surface unit) will of course be equal For mechanical reasons a spherical cell would be expected to be more resistant than a rod The figures of the experiments reveal that the sonic sensitivity was increased particularly during the late lag and the logarithmic stage of growth During the former period the volume area and length diameter quotient all were markedly increased but in the latter period the parameters just mentioned returned to their original values In experiments 1 and 4 the increase of the disintegration effect was greatest viz from 1 42 to 2 93 and from 1 83 to 3 70 log units in seven min respectively These differences mean that the treatment time of bacteria grown for 24 hours had to be more than doubled to obtain a disintegration effect equal to that when bacteria were in the early logarithmic stage of growth In one experiment (exp 3) the plotting of the disintegration curve follows a curve more like that for the size, while in other experiments (1 2, 4 and 5) it is more similar to the quotient curves which express the rod shape of the bacteria In all cases the correlation between disintegration effect and size and shape of the bacteria is not very close

Experiments 2 and 5 deserve special attention In experiment 5 there was an initial decrease in sonic disintegration after the first hours growth but the volume was maintained at the original value and in experiment 2 the disintegration effect was maintained while the volume was considerably increased These data which are not contradicted by those of the other experiments suggest that there might be an increased resistance during the early lag stage when bacteria of the same size are compared This fact is reminiscent of the findings of *Elliker & Frazier* (1938) who demonstrated that E coli is more resistant to heat shock at 53° C for 30 min during initial growth but becomes more sensitive to heat during the late lag and the logarithmic growth stages

A heating effect of sonic oscillation has been demonstrated [illegible]

[illegible] thermometers cannot be responsible for the disintegration since disintegration is easily produced in cooled suspensions The proposition that the very high short lived temperatures (*Bergmann* 1949 suggested 234° C *Noltingk* 1960—40 000° K *Temberj* 1960) locally generated at the cavitation bubble may however be more significant but in this case the distinction between heat and movement may not be clear

It has been observed (*Heden & Wyckoff* 1949 *Sallon & Horne* 1951)

belonged In the present investigation the influence of sonic waves on the disintegration of various Proteus phases (*Belyavin* 1951) was investigated It is of interest that the long filaments of phase C did not occur This might be accounted for by large size of the inoculum Only *when the same strain was used as a smaller inoculum did the shapes* described by Belyavin occur In the investigation by *Malmgren & Hedén* (1947) it was demonstrated that, when the inoculum was increased, the volume variation during the *growth diminished However, even in the* cases when the volume variations were diminished they were statistically significant (Table 1–5) The bacteria in phase C were bigger than those of the other phases (Table 6) This was particularly evident when the cells had been initially harvested after growth on nutrient agar but less obvious after growth in broth It had been noticed earlier (*Dienes* 1946) that the long forms of Proteus were not so easily found in liquid as on solid media This has been confirmed by the experiments described in this paper The length diameter quotient was higher in every experiment immediately after inoculation, after the cells had *grown on solid medium, versus the quotient at the end of the experiments* when growth had gone on 24 hours in broth The difference was often pronounced (Table 1–5) It should also be noted, that phase A bacteria were larger and phase C bacteria smaller after growth in broth than on nutrient agar Proteus grown on the solid medium was generally more easily disintegrated than broth-grown bacteria

The disintegration was estimated by subtracting the log number of intact cells after sonic treatment from the log of the non-sonic treated sample, because it had been found that, within the concentrations used in the investigation, the log number of bacteria decreased almost linearly with time, *regardless of the initial concentration (Fig A)* Earlier investigations are not uniform on this point *Hesselberg* (1958), working with Escherichia coli and Staphylococcus aureus which were ultrasonic treated at smaller effects (400 kC and 2 5–5 watts sq cm), *found that the logarithmic disintegration effect (calculated by viable* count) decreased with increased concentration until it was completely abolished He found that this critical concentration rose as higher ultrasonic effects were employed Similar opinions were expressed by *Stumpf et al* (1946) Other investigators (*Horwood et al* 1950 *Kinsloe et al* 1954 *Marr & Cota-Robles* 1957 *Grun & Steller* 1955 *Davies* 1959) who worked *with higher effects, have claimed that the disintegration,* measured by total or on viable count, is logarithmic regardless of the initial concentration used

In the experiments reported in this paper, the disintegration seemed to proceed logarithmically but just decreasing slightly after prolonged treatment This bend of the curve (Fig A) might be due to a decrease in disintegration effect The suspensions show an increase in viscosity when cell contents are *liberated from the disintegrated bacteria It is* also known that a higher viscosity diminishes the disintegration effect

e g staphylococci and streptococci are to be disintegrated It should also be taken into account in work when comparative studies on the antigenic enzymatic and physico chemical properties of fractions from cells in different physiological states are done

SUMMARY

Proteus mirabilis in its various phases (Belyavin) are more easily disintegrated by sonic treatment during the late lag and the logarithmic growth stages than when not in active growth When the increase in sonic sensitivity is most pronounced the treatment time is reduced to less than half of that needed to obtain the same degree of disintegration when using bacteria in the stationary stage of growth The increase in sonic sensitivity has a certain relation to the size and shape of the cells but the correlation decreases as growth proceeds

It is suggested that the variation in the sonic sensitivity of bacteria during the various growth stages may be due to changes in the cell walls

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that when bacteria are heated above 50° C their cell walls are ruptured. It was further noted that when young cultures (1 hour's growth) were used, cell wall rupture appeared after heating at lower temperatures (*Salton & Horne* 1951). The similarities between the alteration in sensitivity to moderate heat and to sonic oscillation during the lag- and logarithmic stages may therefore be due to a similar mechanism, and possibly dependent on the state of the cell wall.

Investigations which measure the state of the cell wall at different stages of the growth curve are sparse. *Lark & Lark* (1960) outlined Alcaligenes faecalis cell wall synthesis by measuring the incorporation of radioactive methionine and alanine, two essential cell wall ingredients, into the cytoplasm and the phenol soluble and insoble part of the cell wall. They worked with synchronized cultures and found a logarithmically increasing incorporation of these amino acids throughout the growth of the culture. They concluded that no increased cell wall synthesis occurred at the time of division. However, due to a constant volume and an elongation of the cells during the periods between the cell division, they concluded that "it is not necessary to assume that continued synthesis of cell wall material during the interdivision period leads to a thicker cell wall". It would have been of particular interest had they extended their measurements to include the cell wall synthesis after cell division had stopped and the stationary stage had been reached.

Bursting E. coli by the release of nitrous oxide pressure succeeds only when they are in the logarithmic stage of growth (*Fraser* 1951). This occurrence may very well be due to a decreased cell wall strength in such a period. The fact that some kind of change in the cell wall of E. coli does occur in the period of active growth is demonstrated by the altered resistance towards lysozyme (*Repaske* 1958) and the changes in the surface potential when the electrophoretic mobility of cells is determined (*Moyer* 1936).

Shockman et al. (1958) demonstrated that after termination of the exponential growth stage of Streptococcus faecalis, a period of synthesis of cell wall material took place. The contribution of the wall to the weight of the cell increased from 26 per cent during the exponential growth to 38 per cent during the stationary stage without any conspicuous change in the size of the cells. The present investigation makes probable that also the cell wall of Proteus is not so well developed during the logarithmic growth as in the stationary stage.

The increased sonic sensitivity of bacteria during the late lag- and logarithmic stage of growth is not restricted to Proteus species. The logarithmic killing effect of sonic waves for E. coli was about 50 per cent increased during most of the period of active growth (1½–6½ hour after inoculation).

If the increased sensitivity to mechanical disintegration during the active growth is a general characteristic of micro-organisms it will be of great practical importance, particularly when more resistant bacteria,

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CRYPTOCOCCOSIS AS A CAUSE OF PULMONARY ALVEOLAR PROTEINOSIS

By

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Received 24 iii 61

Pulmonary alveolar proteinosis was first described by *Rosen, Castleman & Liebow* (19) in 1958, since when many cases have been reported (1–12, 14–18, 20 22) But as early as in 1951 *one (F L) of us observed* a case with identical changes in the lungs That case, which was combined with widespread cryptococcosis, was published (13) in 1953 with accompanying illustrations (Fig 1) of the lung changes In in investigation of the course of experimental cryptococcosis in mice, *one (F B) of us* noted similar lesions in the lungs It therefore *occurred to us* that cryptococcosis might sometimes be the cause of pul*monary* alveolar proteinosis The findings in the human case and in *the* experimental investigation in mice are described below

REPORT OF HUMAN CASE

The case has been accounted for in *detail elsewhere* (13) Therefore only the main features will be described here

The patient was a 57-year old joiner

Six years before death *he began* to feel tired He had

Autopsy

Gross findings The most striking findings were seen in the right lung which network of fi part of the le

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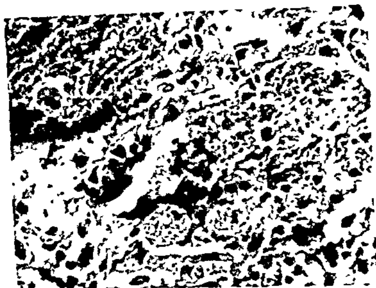


Fig 3

Human case High power view of a pair of alveoli with granular substance Along the walls of the alveoli are large cells with foamy granular cytoplasm Some disintegrating cells of this type with faintly stained nuclei are seen inside the alveoli Periodic acid Schiff stain × 480

white seed- to pea sized foci They resembled productive tuberculous changes, but no caseation was seen

At the tracheal bifurcation was a conglomerate, the size of a goose's egg, of enlarged lymph nodes with a gelatinous cut surface

The skin and the brain contained several cryptococcomas with a gelatinous cut surface

Microscopical findings The mucous material in the lower lobe of the right lung consisted of enormous masses of cryptococci with a small admixture of inflammatory cells The pulmonary tissue was completely destroyed and the masses of cryptococci were enclosed by thin fibrous bands containing lymphocytes plasma cells and scattered histiocytes with pale, foamy cytoplasm

In the pneumonia like foci in the left lung the tissue had preserved its histological structure with thin alveolar walls The alveoli were filled with granular masses that stained faintly blue with hematoxylin eosin (Figs 1–2) They did not stain metachromatically with methylene blue but when stained according to McManus (PAS staining) they took on a red colour and then small droplets [illegible] on examination o [illegible] nuclei were demon- [illegible] cytoplasm similar [illegible] of the alveoli (Fig. [illegible] with foamy cytopl- [illegible] fusiform clefts al [illegible] mucicarmine staining was negative The bronchi in these areas were filled with pus cells, mucous masses and a few scattered *cryptococci In* the neighbourhood of the areas with PAS positive substance in the alveoli were small purulent pneumoniae

EXPERIMENTAL

In the experimental investigation adult white male mice were inoculated subcutaneously in the hind foot with about 15 million yeast-

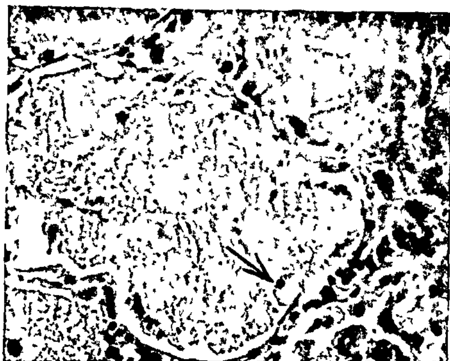


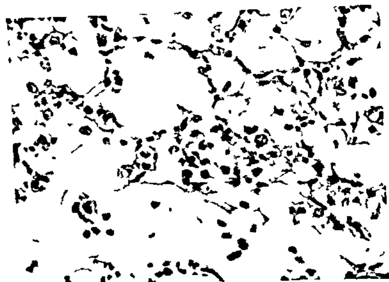
Fig 1

Human case Section of the lung of case published in 1953 Granular masses in the alveoli Arrow indicates a septal cell with granular PAS positive cytoplasm Periodic acid Schiff stain × 300



Fig 2

Human case Low power view showing the alveoli uniformly filled with coagulated granular masses Observe acicular cleft in the granular masses Periodic acid Schiff stain × 1[illegible]0



F g 6
Mouse lung Some alveol are filled with f amy macrophages D wn to the left a c uple of alveoli with granular substance with acicular clefts Other alveoli contain macr phages in d fferent stages of disintegration Htx e sin × 480

cells in a saline suspension from a 4 days old Sabouraud dextrose agar culture of (neoformans The strain had been isolated from spinal fluid from a human case of cryptococcosis (not from the patient in present case report) The animals were sacrificed 10 minutes to 256 days after the injection and histological sections were prepared from the injected foot from lymph nodes and from internal organs From the second to twenty fourth day after injection many of the animals showed disseminated cryptococcosis and culture of the blood gave growth of fungi The lungs showed widespread inflammatory lesions of granulomatous as well as diffuse type with abundant fungi and inflammatory cells The fungi were engulfed by leucocytes and macrophages Later in the course of the infection the inflammatory changes were less marked and more local The number of the leucocytes was reduced but single alveoli or groups of alveoli often showed collections of large foamy macrophages with granulated cytoplasm PAS positive both before and after digestion with diastase (Figs 4 5) Fatty substance was seen in the cytoplasm in frozen sections stained with scarlet red Some groups of macrophages showed faint metachromasia in toluidine blue staining while staining with mucicarmine was always negative The macrophages distended the alveolar spaces and the alveolar walls were thin and contained a few lymphocytes In some areas the macrophages were disintegrated and the alveoli were filled with partly acellular PAS positive substance showing acicular spaces (Fig 6)



Fig 4
Mouse lung Large foamy macrophages filling the alveoli
Grilley's fungus stain × 750

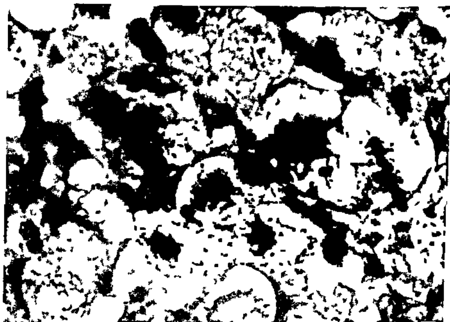


Fig 5
Mouse lung High power view of macrophages with granular PAS positive cytoplasm
Periodic acid Schiff stain × 12[illegible]

changes Our assumption (13) that the content of the alveoli had emanated from disintegrated cryptococci was therefore purely hypothetical, but seems to be supported by our subsequent findings in experimental cryptococcosis in mice As pointed out above, regression of the cryptococcal infection is accompanied by the appearance of large alveolar phagocytes (Fig 4), containing PAS positive granules It is tempting to assume that these cells owe their cytological characteristics to phagocytosis of disintegrating cryptococci These phagocytes closely resemble the "septal" cells observed in our human case (Fig 3) and in other cases on record It is true that mice never showed a fully developed picture of alveolar pulmonary proteinosis with large amounts of loose amorphous masses in the alveoli but only a stage with similar substance located mainly intracellularly However, the changes appeared to be so suggestive (Fig 5) that they deserve further investigation for any relationship between the alveolar pulmonary proteinosis and cryptococcosis It is, of course, by no means suggested that cryptococcosis should always be responsible for alveolar pulmonary proteinosis but simply that it might be worth while considering the possibility of cryptococcosis being the causal factor in some of the cases of alveolar pulmonary proteinosis, which despite its uniform histopathological picture might very well be of multiple origin In this connection it might be mentioned that the lung changes have been found in association with such different diseases as thrombocythaemia (12) and Weber Christian's panniculitis (20)

SUMMARY

A case of cryptococcosis complicated with alveolar pulmonary proteinosis is reported The case was originally published in 1953 but at that time only casual mention was made of the illustrated lung changes, which were tentatively ascribed to aspiration of disintegrating cryptococci

In an extensive investigation of experimental cryptococcosis in mice, regression of pulmonary cryptococcosis was found to be accompanied by collections in the alveoli of large macrophages containing granular PAS positive substance resembling that found in alveolar pulmonary proteinosis

[illegible]

be [illegible]

so [illegible] pulmonary proteinosis, which might be a disease of multiple aetiology

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One to two weeks after the injection the subcutaneous tissue at the site of the inoculation contained collections of macrophages with the same *tinctorial properties as the macrophages in the lung lesion*

COMMENTS

Pulmonary alveolar proteinosis appears to be a histopathological entity whose aetiology is obscure It is remarkable that *Rosen, Castleman & Liebow* (19) were able to collect as many as 27 cases for their first publication Since then some 20 cases have been reported (1–11, 14 18 21–22) In addition, 2 cases with similar lung changes have been described in association with different diseases (12, 20) It is beyond *doubt that the case outlined in the present investigation (13)* satisfies the histological criteria for the diagnosis The PAS-positive granular substance filling the alveoli was of typical appearance, as were the "septal" cells in the alveoli In addition, the granular masses were interspersed with acicular crystals Moreover, purulent pneumonitic were seen *as well as large areas where the lung tissue had been destroyed by* enormous masses of cryptococci Since the granular masses in the alveoli stained in the same way (PAS-positive) as the capsules of the cryptococci, it was claimed in a previous paper (13) that "the changes are probably sequelae from the aspiration of mucous matter that is probably shed by *disintegrating cryptococci To our knowledge* no such observations are on record" Of all the published cases of alveolar pulmonary proteinosis, only one seems to have been complicated by cryptococcosis (case 24 in *Rosen et al* (19)) Judging from the data given, the cryptococcosis in that case appeared later than the alveolar pulmonary proteinosis, "cultures of biopsy material yielded no growth on ordinary mediums" But this does not, of course, exclude the possibility of widespread cryptococcal lesions in other parts of the lungs *Bronchial washings cultured on Sabouraud's medium had, however,* formerly been negative At autopsy 14 months later "the characteristic changes of cryptococcosis were seen in all lobes of the lungs" The report does not say whether alveolar pulmonary proteinosis had also been demonstrable at autopsy The authors thus assume that the cryptococcosis was secondary to alveolar pulmonary proteinosis in their case, particularly since the patient had been treated with large doses of cortisone which might have reduced his resistance against infections In 3 other cases (2, 10, 19), *which were complicated by fungal infections* (in 2 cases of nocardiosis in 1 of mucormycosis) the history also argues for the fungal infection having been secondary to alveolar pulmonary proteinosis which is believed to pave the way for various pulmonary infections In our previous case it was not possible to know whether *alveolar pulmonary proteinosis* had preceded the cryptococcosis, since biopsy had not been done and no specimens had been cultured for fungi before roentgenological demonstration of the lung

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ON THE INCIDENCE OF SEX CHROMATIN IN HUMAN PROSTATIC CANCER

By

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Received 14. ix. 61

The Morphology of Sex Chromatin in Normal and Cancer Tissue

Moore & Barr (13) in 1957 examined the size and appearance of sex chromatin in tumours. By measuring it was found to be 0.8 × 1.3 μ in average. In a single case the sex chromatin body was measured to 1.1 × 2.0 μ which is a little more than usual in normal tissues. *Orsi & Ritter* (14) examined tissue cultures of carcinoma cells from breast cancer and found sex chromatin of normal appearance and size in 60 to 64 per cent of the cells. In benign tumours *Moore & Barr* (12) found the average size of sex chromatin as 1.2 × 0.8 μ. *Miles* (8) examined sex chromatin in normal tissue and cancer in explanted cultures, using the Klinger method for staining and found the sex chromatin to be a planoconvex mass as described by several writers. Often it was found as two separate parts, very close together, an observation confirmed by *Klinger* (5) who now and then in normal female cells observed a pale area in the center of the body or adjacent to the cell membrane. By examination of 11 tissue cultures of various cancer cells of female origin *Miles* (8) counted a sex chromatin mass in up to 66 per cent of the cells. In 10 cultures of tumour cells from male tissue the sex chromatin per cent was observed to be from 0 to 4 per cent. Various tumours showed no difference from the sex chromatin observed in normal cells. The authors quoted have not been able to demonstrate differences in morphology between sex chromatin in normal cells and in tumour cells.

Prostatic Tumours

A number of authors have presented evidence to the effect that it is possible to determine the sex of various human tissues by counting the sex chromatin content (1, 10, 11, 13).

Coutts, Silva Inzunza & Coutts (2) in 1956 reported on studies of 78 cases of prostatic enlargement. 39 have been found containing female sex chromatin in more than 10 per cent (average 25 per cent) of nuclei, which according to the writers' experience is characteristic for tissues of female origin. Furthermore 2 cases among 12 prostatic carcinomas

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RESULTS

As it is seen from Table 1, the average content of sex chromatin is 3 per cent in tumour cells and 2 per cent in the control material The sex chromatin percent varies between 1 and 9 per cent in the tumour cells and between 0 and 4 per cent in the smooth muscle cells

DISCUSSION

It appears that some writers have observed cases of prostatic cancer in which the incidence of sex chromatin is so high that from a cytological point of view they considered these tumours to be of female sex This has not been confirmed by the present material The highest sex chromatin content found was 9 per cent in a single case, and no case showed such discrepancy between the tumour and the control count that the former could be considered as of a sex opposite to that of the control tissue

These results are in agreement with the findings of some previous writers (3, 4, 7, 13) Since the present material was carefully selected, with a view to avoid the effect of autolysis it may be assumed that various earlier reports of high incidence of sex chromatin in prostatic tumours may be due to misinterpretation of abnormal tumour nuclei, since tumour cells often contain several chromatin bodies similar to sex chromatin Especially in carcinoma cells of the prostate numerous nucleoli often appear near the nuclear membrane (7), which in phase contrast microscopy may simulate sex chromatin With the anaplasia and hyperchromasia of tumour cells also insufficient technique may [illegible]

[illegible] the nucleus, for instance Feulgen, Klinger's thionin or Lennox's ribonuclease gallocyanin stain

Fig 1 shows a displacement towards the right of the tumour group in comparison with the control group This suggests less accurate results from the counting and interpretation of sex chromatin in tumour cells than in normal cells, also with the technique of the author A nuclear chromatin body must be counted as positive in male tissue if a similar mass would be counted as sex chromatin on the [illegible]

[illegible] of cells with very anaplastic and bizarre nuclei should, therefore, contain a high per cent of sex chromatin like nuclear bodies, the interpretation of which demands a well trained observer

were also classified as female, containing 25 *and* 35 *per cent* sex chromatin Coutts et al used phasecontrast microscopy of fresh, unfixed material

In 1959 *Lehman, Hodges & Oyamada* (6) examined 35 prostates, 10 of which were carcinomas Among the 25 benign enlarged prostates 3 were *observed* to contain 20, 27, and 37 per cent sex chromatin and one carcinoma contained 32 per cent sex chromatin The staining used was ordinary haematoxylin

Among 18 undifferentiated tumours from various tissues from male patients *Tavares* (15) reported 2 cases considered as female since they contained 32 per cent sex chromatin

Using phase contrast technique *Montenegro-Ortiz & Silva-Inzunza* (9) described several malignant tumours as being of opposite sex to that of the host-organism

Other authors, however, have not been able to verify the abovementioned findings by examining prostatic tumours

Twenty cases of prostatic carcinomas were *studied by Heinz & Ehlers* (3, 4) who in no case counted more than 0 5 per cent of cells showing sex chromatin *Lennox et al* (7) examined 71 patients with cancer or enlargement of the prostate, and in neither case any discrepancy was found between the sex of the patients and the sex of tumours *Three cases* of adenocarcinomas of the prostate described by *Barr & Moore* (13) showed the same results

MATERIAL AND METHODS

Tissues from 24 cases of carcinoma of the prostate were examined for sex chromatin content by the Feulgen technique Histologically and clinically all cases were typical carcinoma Only preparations without signs of autolysis were applied In each case *400* cells were examined and as a control of the cellular sex of the patient 100 cells of smooth musculature were counted in the same section The average findings are listed in Table 1

TABLE 1

The Incidence of Sex Chromatin in Tumour Cells from 24 Cases of Prostatic Cancer in Per Cent As Control Serves the Incidence of Sex Chromatin as per cent in Cells of Smooth Musculature Counted in the same Sections

		Sex chromatin in tumour cells as average per cent of 400 cells per case		Sex chromatin in smooth muscle cells as per cent of 100 cells per case
No of Cases	7	1	4	0
	7	2	6	1
	5	3	8	2
	3	5	5	3
	1	6	1	4
	1	9		
Total	24	Average 3	24	Average 2

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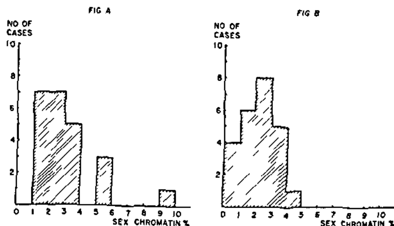


Fig 1

A) Per cent of Sex Chromatin in tumour cells of 24 cases of Prostatic Cancer given as per cent of average

B) Per cent of *Sex Chromatin in smooth musculature in same cases and sections* as Fig A

SUMMARY

Some authors have reported differences in the occurrence of sex chromatin between the tissues of tumours and those of their hosts Others have been unable to confirm these results as far as prostatic cancer is concerned On the examination of 24 tumours carefully selected with a view to clinical verification and to their state of preservation the present author found an insignificantly higher per cent of sex chromatin among tumour cells than among musculature in the same section This *difference, however, is ascribed to misinterpretation of* findings due to nuclear abnormalities in tumour tissue, and it is far too small to suggest any differences in cytological sex between prostatic tumours and their hosts

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8 ... al and Cancerous Human Tissues

TABLE 1

Distribution of the Islet Numbers (Upper Part) and Volumes (Lower Part) into Different Size Classes Expressed in per 1000. For each Size Class (X_2–X_{12}) the Mid Class Value for the Islet Diameter in μ has been given within Brackets

Case	X_2 ([illegible])	X_3 ([illegible])	X_4 (85.[illegible])	X_5 (101.[illegible])	X_6 (125.0)	X_7 (156.3)	X_8 (187.5)	X_9 (218.8)	X_{10} (250.0)	X_{11} (281.3)	X_{12} (312.5)
					Islet number						
1	278.7	236.0	167.3	144.5	123.7	34.8	9.1	3.5	2.5	–	–
2	241.3	216.6	163.1	131.4	150.7	64.0	17.4	5.7	0.7	–	–
3	282.6	230.0	174.9	137.7	118.3	34.3	12.4	6.7	2.6	–	–
4	352.9	257.0	170.3	103.9	79.6	26.0	9.1	1.3	–	–	–
					Islet volume						
1	31.2	91.8	118.6	169.1	270.4	148.2	67.2	40.7	42.8	–	–
2	36.1	[illegible]8.6	94.3	125.3	284.4	222.4	104.4	54.1	10.4	–	–
3	49.6	85.7	118.5	154.0	247.1	139.9	87.3	73.7	44.1	–	–
4	83.7	129.0	15[illegible].0	157.1	224.6	143.2	86.4	20.0	–	–	–

It should be noted that the first four size classes (X_2–X_5) have only half the breadth of the remaining classes

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THE FREQUENCY DISTRIBUTION OF THE NUMBER AND VOLUME OF THE ISLETS OF LANGERHANS IN MAN

3. *Studies in Diabetes of Early Onset, Insuloma and Acromegaly*

By

BO HELLMAN and LENNART ANGERVALL

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The pancreatic islet organ in diabetes of late onset was found to be characterized by a striking regularity, except when the islets were hyalinized (*Hellman* 1961 a). The volumetric islet distribution in these cases showed the same mathematical relationship to the islet diameters as previously had been found to be valid for non-diabetics (*Hellman* 1959 a, b).

Patients developing diabetes during the growing period have low total weight of B cells, whereas those developing diabetes after maturation have higher volumes (*Maclean & Ogilvie* 1955). Besides diabetes of early onset, insuloma and acromegaly are examples of endocrine disorders in which morphological islet changes are reported to occur (*Hultquist* 1946, *Ferner* 1951, *Simpson* 1959). Hence it seems useful to have knowledge of the size frequency distribution of the islets also in these cases and compare the findings with those in diabetes of late onset and in non-diabetics. The results of such an analysis is presented in this paper.

MATERIAL AND METHODS

The analyses were carried out on the pancreatic tail. In Case 2 material was resected at laparotomy, but in the remaining cases the tip of the tail was excised at autopsy. After fixation in Bouin's solution, dehydration and clearing the pancreatic specimens were embedded in paraffin and cut into 7 µ thick serial sections.

The islet distribution analyses were performed on every 44th section. After staining with *Gomori's* chrome hematoxylin phloxine method the sections were systematically scanned for islets, whose sizes were classified by comparing their projected images at 240 × magnification with circles and ellipses of known areas. The relation between the mid class value for the islet diameters in µ and the class number can be seen in Table 1. From each pancreas sufficient sections were analysed to bring the number of islet section surfaces classified to at least 500. For conversion of the apparent size distribution obtained in this way to the actual size distribution



Fig 2

Silver impregnated paraffin section from the pancreas in Case 2 The picture illustrates that this pancreas is very rich in islets of Langerhans the total volume of which is considerable In spite of the presence of an insuloma the percentage frequency of the argyrophil cells in the islets of Langerhans is not higher than normal

other islet cells The percentage frequency of argyrophil islet cells was found to be within the normal range

Case 2

The patient was a woman of 47 The history was characteristic of insuloma and hypoglycemia occurred spontaneously *Whipple's* triad was present When other causes of spontaneous hypoglycemia—such as liver insufficiency and adrenocortical atrophy—had been eliminated it was decided to operate The excised portion of the pancreatic tail weighed 12 7 g and included a rounded well circumscribed [illegible]

[illegible] in collagen capsule There was no manifest tumour growth within or outs[illegible]
[illegible] of the tumour there wer[illegible]
being more or less rounded and so[illegible]
was completely dev[o]id of argyrop[illegible]
outside were distinctly silver impreg[nated] (see Fig 2)

Case 3

The patient was a woman of 42 who had been strumectomised for hyperthyreosis in 1936 She had been taking insulin for diabetes since 1945 when she was 27 Grave retinopathy with diminished visual acuity and signs of neuropathy were discovered in 1955 In January 1959 the nonprotein nitrogen level was slightly elevated The patient died in March 1960 of uremia and circulatory insufficiency



Fig 1

Silver impregnated paraffin section from the insuloma in Case 2 with surrounding pancreatic parenchyma In the lower part of the Figure it can be seen some of the epithelial tumour, which is completely devoid of argyrophil cells The tumour is well delimited by a capsule from the surrounding tissue In the upper part of the Figure there are two islets of Langerhans containing a number of distinct argyrophil cells

the equation system derived by *Wicksell* (1925 1926) was used The procedure for this was identical with that used in a previous study (*Hellman* 1959 a)

The morphological findings, including the general appearance of the islets are described in the section Case Reports Except for the chrome-hematoxylin phloxine stain according to *Gomori* the latter observations on islet cytology were based on the silver impregnation method on thin paraffin sections, as described by *Hellerström & Hellman* (1960) In assessing the frequency of silver-positive cells regard was paid to the fact, that there is an apparent over representation of the argyrophil cells, see *Hellman* (1961 b)

CASE REPORTS

Case 1

The patient was a man of 44 with acromegaly since age 18 There were no signs of diabetes mellitus During the period 1940 to 1947 the patient received a series of intensive roentgen irradiations towards the pituitary body After this therapy the thyroid markedly enlarged but there was no definite signs or symptoms of abnormal thyroid function In 1960 the daily urinary gonadotropin output was normal about 6 5 M U that of 17 hydroxycorticosteroid 4 4 mg and that of 17 ketosteroid 1 5 mg The patient died of heart failure in March 1960

Autopsy (Uppsala 183/60) was performed 15 hours after death Apart from the skeletal changes typical of an acromegalic it disclosed a shrunken pituitary body in a sella turcica about twice as large as normal Partly located intrathoracically the thyroid weighed 800 g and contained partly necrotic adenomata The adrenals and testes were atrophic

Histological examination of the pituitary showed a highly regressively changed colloid rich adenoma with chromophobes and sparsely granulated eosinophils (mixed adenoma) There was also a small remnant of anterior pituitary lobe with normal

The pancreas (weight 120 g)

ated centrally were markedly

times the nuclear diameter of

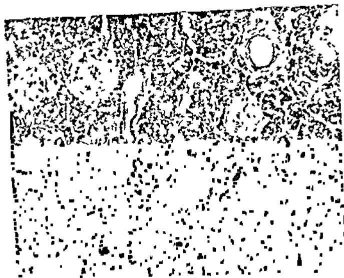


Fig 2

... The picture illu... ie total volume of ... a, the percentage ... not higher than normal

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... pale blu... con- side...

... (see Fig 2)

Case 3

The patient was a woman of 42 who had been strumectomised for hyperthyreosis in 1936 She had been taking insulin for diabetes since 19...

Autopsy (S S 426/60) which was performed 13 hours after death disclosed a remarkably small pancreas Histological examination The kidneys exhibit diffuse and nodular glomerulosclerosis of *Kimmelstiel Wilson s* type and pronounced arteriolohyalinosis The *pancreas displayed marked arteriolohyalinosis The exocrine* parenchyma was fibrotic and atrophic There were very few islets of Langerhans

Case 4

The father contracted diabetes at age 40 a sister died of diabetes at 18 months The patient s diabetes was discovered in and had been insulin treated since 1936 when he was 17 years of age Signs of neuropathy and retinopathy were found in 1947 In 1953 he underwent subtotal thyroidectomy for hyperthyreosis and subsequently received 0 1 mg twice daily *of Levoxin (1 thyroxin) Since 1953 there* has been progressive renal insufficiency He was admitted to hospital in January 1960 with grave anemia and mild uremia and died of uremia in August 1960

Autopsy (S S 778/60) was performed 78 hours after death and disclosed a small pancreas weighing 22 g Histological examination The kidneys displayed diffuse and nodular glomerulosclerosis of *Kimmelstiel Wilson s* type and pronounced arteriolohyalinosis The pancreas was atrophic and contained very few islets

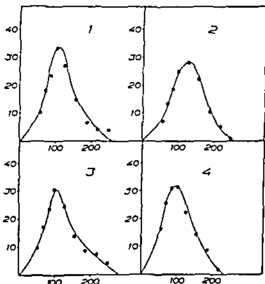


Fig 3

The percentage volume distribution of the islet tissue in relation to the islet diameters for the different cases

RESULTS

The distributions of the numbers of islets into different size classes expressed per 1000, are given in the upper part of Table 1 It is evident that the numerical distribution curves in all cases were highly asymmetric, since the number of islets increases progressively with decreasing islet diameter In reading the Table it must be borne in mind that the class width in the first four classes was only half as great as in the remaining ones In Table 1 the mid class value for the islet diameter in μ is given in brackets for each class number

From the numerical size distribution one can calculate how the total islet volume is distributed in relation to the islet diameter This volumetric size distribution in the cases studied is presented expressed per

1000 in the lower part of Table 1 The main part of the islet volume was constituted by the medium sized islets, while the very numerous small islets contributed an approximately similar amount to the islet volume as did the fewer larger islets In Fig 3, where the values have been plotted graphically with allowance for the differences in class width it is evident that the volumetric islet distribution curves in three of the cases have a clear symmetrical form In Case 3 a displacement of the peak of the curve to the left caused slight asymmetry

DISCUSSION

Earlier studies in rats and mice have shown, that the pancreatic islet organ attempts to retain the balance between the number of large and small islets Thus in spite of great changes in the total islet volume and the B/A cell number ratio, the volume distribution curves retained their typical symmetrical form (*Hellman* 1959 c, *Hellman et al* 1961) The islet organ in man seems to be no exception in this respect, since a symmetrical relationship was also found in diabetic adults, in whom the disease first became manifest at an old age (*Hellman* 1961 a) In addition w e f n l in th - -t

... islet organ It is further of interest that, in an acromegalic intensively treated and having signs of persisting pituitary function, the same symmetrical relationship could also be demonstrated

It seems reasonable to judge the slight deviation from symmetry observed in Case 3 more as an expression of the great technical difficulties in doing the analyses in this pancreatic gland than showing a true biological divergence from the main principle The islets in the two diabetics and especially in Case 3, were actually extremely few and difficult to ...

poin

in it ... diabetes of early

onset

It was apparent from systematic tests, that the symmetrical form of the curves is not influenced by the fact that we have used both fresh material (Case 2) and such taken as long as 78 hours after death (Case 4) or have fixed for different lengths of time (*Hellman* 1959 a) On the other hand this of course means, that it is not possible to compare the absolute islet size in our cases with each other or with the values given earlier for non diabetics or for persons, where the diabetes was manifest at adult age

The finding that the total islet volume was regularly arranged even in those cases suffering from endocrine disorders associated with morphological changes in the islets, can probably be used for simplifying the determinations of the total islet volume in man For example,

it may be mentioned that in rats merely counting the number of islet section surfaces exceeding a certain size is enough to obtain an estimate of the total islet volume (*Hellman* 1959 d, *Petersson et al* 1960)

SUMMARY

The actual size distribution of the islets of Langerhans was studied in one subject with an insuloma, one treated acromegalic showing persisting pituitary function, and in two individuals with diabetes of early onset Though these disorders are associated with morphological changes in the islets, the islet organ still retained the same regular arrangement as was previously found to be characteristic of non diabetics and diabetics of late onset A slight deviation from symmetry in the volume distribution curve in one of the diabetics was judged more as an expression of the technical difficulties in doing the analysis than being of true biological existence The observations are compared with the islet distribution analyses earlier performed in different species including man

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FROM THE NEURO-SURGICAL DEPARTMENT OF THE BISPEBJERG HOSPITAL CHIEF BENDT BROAGER—THE PEDIATRIC SURGICAL DEPARTMENT OF THE STATE HOSPITAL (RIGSHOSPITALET) CHIEF C C WINKEL SMITH—THE NEURO PATHOLOGICAL LABORATORY OF THE STATE HOSPITAL (RIGSHOSPITALET) CHIEF ERNA CHRISTENSEN COPENHAGEN

CONGENITAL MALIGNANT MESENCHYMAL TUMOURS IN A TWO MONTH-OLD CHILD

By

ERNA CHRISTENSEN, KNUD HØJGAARD and C C WINKEL SMITH

Received 3 v 61

Malignant mesenchymal tumours with generalized metastases represent a rare occurrence in newborn children In 1940 *H G Wells* published an analysis of previously reported cases, a total of 30 sarcomas including 9 fibrosarcomas Generalized metastases to various organs were seen only in 3 of the reported cases In addition the histories of a few cases of congenital fibrosarcomas have been reported *M L Deyfuss & Clifton Forge* (1949) described a fibrosarcoma in the foot *J O Williams & D Schrum* (1951) and *D Robb* (1958) each described one case of congenital fibrosarcoma with metastases

Hence with a view to the rare occurrence of such tumours we have considered the following case of general interest

CASE HISTORY

Pediatric Department The State H [illegible]

The patient a 2 mon [illegible]

[illegible]

[illegible] surgical department the child was in a physically satisfactory condition Turgor of the skin and sucking were normal The circumferences of the head and the fontanelles were normal On the right side of the neck 1 2 cm behind the angle of the jaw a solid rather irregular tumour, the size of a walnut was found

The neurological examination [illegible] paresis of the upper [illegible] mities was less prono [illegible]

Radiographic exam [illegible] physeal region of all [illegible]

of about 1–2 cm was found to be completely devoid of calcium The line of demarcation of this area was convex against the diaphysis A narrow, striated calcification occupied distally a position adjacent to the epiphyseal line In the metaphyseal calcium deficient areas the bone had become distended This picture was interpreted as syphilitic osteochondritis (Fig 1)

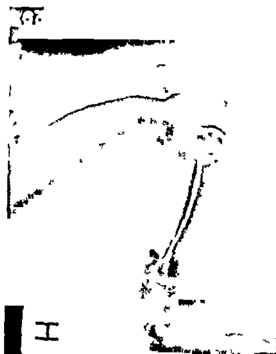


Fig 1
In Fig 1 is seen the right leg examined by X ray A similar picture was found in all the other long bones see text

Among the numerous laboratory findings the following are of particular interest
Haemoglobin percentage 75 72 Micro Sedimentation rate 20 5 mm
Wassermann and T P I (Nelson) reactions Negative
Serum calcium 8 4 mg per cent Serum phosphorus 6 5 mg per cent

On November 13, 1959 when the child was about one month old she was referred to the pediatric surgical department for further examination Still her general condition was rather good except for slight paleness The right sided cervical tumour had remained stationary and was still the size of a walnut At this stage however the right arm and the right leg were almost paralysed The left arm was markedly paretic the left leg was slightly paretic and the tonicity was reduced in all four extremities Within the following month the paralysed lower extremities became spastic

When the child was 5 weeks old biopsy of the tumour revealed sarcoma Since admission to the neuro surgical department one month earlier tumour seemed to have ceased growing About 4 weeks after the biopsy exploration showed tumour behind the right cervical vascular sheath displacing it forward Directly behind and in close apposition to the tumour the brachial plexus was noted and dissected free Tumour growth had invaded the spinal canal and it was decided to abstain from radical extirpation and do nothing except excise tumour tissue outside the spinal canal

Postoperatively the child became dyspnoeic and cyanotic Despite tracheotomy and assisted respiration death occurred less than 24 hours after the operation

The excised tumour tissue was of a whitish colour resembling fish meat The histological examination of the tumour revealed tissue much resembling that de

scribed in the autopsy report Several areas however were highly polymorphocellular and polymorphonuclear and the nuclei contained one or more nucleoli The gross necrotic areas were found to consist of beginning regeneration of the connective tissue

Post mortem Examination

Gross examination Autopsy showed almost total pulmonary atelectasis The right side of the neck was the side of an irregular outlined tumour invading the spinal canal through a mutilated vertebral arch in the cervical spine A 1½ × 3 cm area of cervical medulla, external to the dura mater, was coated by tumour tissue The tumour tissue was greyish red, solid and uniform Several nerve fibres were seen to protude from the tumour tissue into the spinal canal but gross examination revealed no medullary involvement No enlarged lymph nodes were seen The right humerus and the right femur were dissected free thus

Histological examination Examination of slides prepared from the frontal cortex, the striate body, the occipital cortex, the mesencephalon and pons the medulla oblongata, the cervical medulla, and the cerebellum, discloses normal development of the central nerve system in full accord with the age of the child, stratification, differentiation, and myelination are normal Universal, marked edema and stasis are observed, occasionally accompanied by fresh, perivascular haemorrhages No tumour tissue is present

In a slide prepared from paravertebrally excised tumour tissue the sympathetic trunk is covered and compressed by tumour tissue (Fig 2 A) Some of the nerve cells are well preserved, while others have degenerated The perineurium is not involved The tumour tissue is found to consist of spindle shaped, cytoplasm rich, pale cells with oval, usually large and pale nuclei Isolated mitoses are also seen The line of demarcation between the cells is blurred but longitudinal ramifications from the cells are discernible The ramifications are arranged in strands intersecting each other in different directions Vessels are few in the tumour tissue and the vascular walls are not involved The tumour tissue is intersected by strands of fibrous connective tissue of varying thickness In one area of the slide the vertebral corpus is found to be destroyed by tumour invasion With *the exception of the sympathetic trunk* no nerve tissue is encountered

A slide prepared from the intraspinal tumour shows tissue located external to but without involvement of the dura mater This tumour compresses the spinal ganglions and the medulla At the sites in which compression is most marked the medulla has become

... subdural radices in close apposition to the

of about 1–2 cm was found to be completely devoid of calcium The line of demarcation of this area was convex against the diaphysis A narrow, striated calcification occupied distally a position adjacent to the epiphyseal line In the metaphyseal calcium deficient areas the bone had become distended This picture was interpreted as syphilitic osteochondritis (Fig 1)

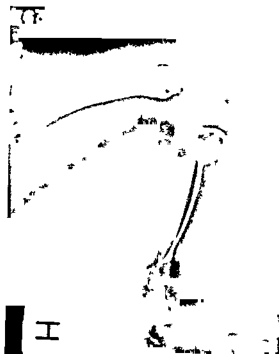


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The excised tumour tissue was of a whitish colour resembling fish meat The histological examination of the tumour revealed tissue much resembling that de

tumour show degeneration of the myelin sheaths Cellular structure resembles that previously described

In slides prepared from the heads of the right femur and the right humerus the tumour tissue will be seen to be of the same nature as tissues hitherto discussed (Fig 2 B–D) Tumour tissue fills up the medullary cavity in the epiphyseal and the metaphyseal areas, penetrating the hyaline cartilage and invading the bone tissue without actual infiltration In several areas the tumour is encircled only by a thin border of solid bone In other areas a little spongiform bone tissue remains and here close to the tumour, are seen osteoblasts No nerve tissue is found in the tumour

DISCUSSION

Histological examination has thus shown several separate malignant tumours all of which are similar with respect to uniformity, polymorphocellularity and polymorphonuclearity, mitoses and amitotic division of cells The cells are oval, rather large, pale, arranged in strands intersecting each other in different directions The nuclei are large and oval No doubt this must be a case of multiple fibrosarcomas

The present study fails to answer the question of whether this case represents multiple primary mesenchymal tumours or metastases to all epiphyseal regions from the cervical tumour The fact that the tumours are present only at the described sites makes the latter improbable since sarcomas are expected to metastazise to a number of organs, *e g* the lung and the liver

SUMMARY

A case of congenital fibrosarcomas in a 2 month old girl is described [illegible] on the right side of the neck and in the epiphyseal regions of the long bones The tumour of the neck was invading the spinal canal coating medulla spinalis

ADDENDUM

After finishing the paper we have been informed of two more articles of the subject

A P Stout Juvenile Fibromatosis Cancer, 7 953, 1954

A Survey of 42 cases in infants and children but in this paper multiple bone tumours do not occur

W B Ober, J A Smith & F C Rouillard Congenital Sarcoma Botryodes of the Vagina Cancer, 11 627, 1958



Fig 2

A slide prepared from tumour on side of the neck with spinal ganglion and nerve root coated by tumour tissue Mallory aniline blue orange G staining × 35 B tumour tissue from head of the right humerus The limit between bone and tumour Haematoxylin eosin staining × 140 C same slide as B × 350 D tumour tissue from head of right femur Limit between bone and tumour Mallory aniline blue orange G staining × 20

tumour show degeneration of the myelin sheaths. Cellular structure resembles that previously described.

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SUMMARY

A case of congenital fibrosarcomas in a 2-month old girl is described. On the fourth post-natal day a hard nodule on the right side of the neck and a mild paresis of the right arm were noted. The symptoms were rapidly aggravating and the child died two months old. By autopsy one found fibrosarcomas on the right side of the neck and in the epiphyseal regions of the long bones. The tumour of the neck was invading the spinal canal coding medulla spinalis.

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Report of two cases from infants Those children are still living and they have no symptoms of metastases or multiple tumours

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PNEUMATOSIS CYSTOIDES INTESTINALIS

By

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Pneumatosis cystoides intestinalis (p.c.i.) is a relatively rare condition characterized by multiple gas-filled cysts in the intestinal walls.

The term was coined by *Mayer* in 1825, but the condition had been described as early as 1730 by British and French authors. Changes of the same type, particularly in pigs, have long been known to veterinary surgeons and butchers.

Similar alterations have been observed, though less frequently, in the human stomach, gallbladder, ligaments, peritoneum, mesenterium, omentum, urinary bladder and vagina. These changes have been described under such names as pneumatosis cystoides intestinalis, enteric pneumatosis, abdominal gas cysts, intestinal interstitial emphysema, gastritis, enteritis emphysematosa, etc.

One of the most complete compilations of p.c.i. is that published by *Koss* in 1952 (5). He reported 213 cases in patients of all ages, mostly between 30 and 50 years. He found the condition to be about three and a half times as common in males as in females.

134 of the patients had cysts in the small intestine, and in 9 of them the colon was also involved. In 73 per cent of the cases the condition was associated with obstructive changes in the region of the pylorus, usually with ulcer.

In 34 the changes were seen only in or near the caecum. In 14 of these cases the disease could be classified as primary owing to the absence of any other intestinal disease.

Only in 13 cases did *Koss* (5) find the changes to be confined to the other parts of the colon only, usually the left colon, while *Smith et al.* in 1958 (19) were able to collect 31 cases of this type. When the disease was limited to these parts of the colon there was seldom any associated intestinal disease (16, 19). It was equally common in both sexes and the onset was somewhat later than that in other parts of the intestines. Of the less common associated diseases, mention might be made of cancer, enteritis of specific or non-specific type, ulceration, etc.

CASE REPORTS

The pathology of the disease is apparent from the following illustrative cases.

Report of two cases from infants Those children are still living and they have no symptoms of metastases or multiple tumours

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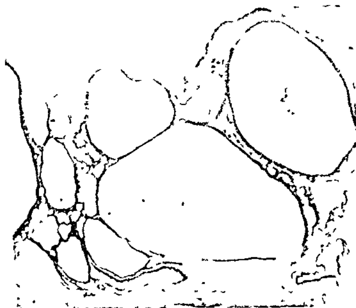


Fig 2
Wall of colon with mucosa at top Empty cysts of varying size in submucosa and intramuscularly × 25



Fig 3
Intramuscular cyst, lined partly with endothelium with giant cells × 83



Fig 1

Intestines with caecal pole in bottom left hand corner Cysts within the sigmoid colon in bottom right hand corner (slit up) and within two parts of the transverse colon (at top)

Case 1 —A 62 year old woman who had had severe bronchial asthma for 20 years developed schizophrenia at 47 years for which she was admitted to a mental hospital on several occasions during the last 8 years of her life During this period she had sometimes had meteorism and occasionally diffuse epigastric pain as well as pain suggestive of cholecystitis Roentgen examination revealed no abnormalities of the stomach or biliary tract During the last few years she had had spells of lip cyanosis and a feeling of tension in the chest The patient died fairly suddenly during an infection of the upper respiratory tract

Postmortem examination (69/1959 Pathological Institute Lund) revealed acute cardiac failure with moderate general enlargement and dilatation of the heart which was flabby and showed signs of myocardial fibrosis Pulmonary oedema pleural transudate and acute congestion of the liver and spleen were also observed The lungs also showed bronchiectasis widespread lung emphysema and histologically a moderate brown pulmonary induration and purulent tracheobronchitis as well as haemorrhagic bronchopneumonia The liver contained multiple cavernous haemangiomas

The abdomen and the intestines were distended with gas The sigmoid flexure and transverse colon formed two large semicircular loops In these loops in the neighbouring parts of the ampulla recti and within a 10 cm length of the splenic

bladder showed no gross or microscopic signs of acute or chronic disease, and the bile ducts contained no concrements

Histological examination of the changed parts of the colon showed mostly thin walled cysts of varying size in the submucosa and subserosa as well as in the muscularis (Figs 2-3) They were lined completely or partly by occasionally multilayered endothelium with eosinophilic cytoplasm and small round nuclei with dense chromatin Here and there multinucleated giant cells were seen (Fig 4) The wall was built up of a collagen rich connective tissue with bands of lymphocytes In some areas slit shaped cysts were seen, and some of them were filled with desquamated endothelium and giant cells (Fig 5) Some of the cysts had no endothelial lining The mesenterium with the lymphnodes jejunum and ileum as well

and nocturnal attacks of dyspnoea with wheezing respiration Severe pulmonary emphysema and bronchiectasis were diagnosed The respiratory trouble was interpreted as cardiac asthma Spirometer

increased residual fun

signs of poor alveolar v

constipation and flatul

of the colon but the

Repeated roentgen examination the following year was incomplete At 75 years cardiac infarction occurred to which she soon succumbed

Postmortem examination (516/1960 *Pathological Institute, Malmö*[1]) revealed nothing noteworthy in the stomach except a scar after an ulcer The entire colon and mesocolon showed subserous and submucous air filled cysts Some of the cysts were densely crowded and on the serosa side they were up to the size of a fist, on the mucosa side they were walnut sized No ulcerations were seen in the intestinal mucosa which appeared to be atrophic and reddened and

missing or replaced by multinuclear giant cells. Giant cells and pale epitheloid cells were also seen interstitially as granulomas or in slit shaped spaces The changes were surrounded by moderate cell infiltration

The postmortem examination also revealed hypertrophy and dilatation of the left half of the heart The aorta and coronary vessels

ron pigment in

In these cases, then, the examination showed gross and microscopical changes fitting in with the diagnosis of p c i In view of the localization of the changes, the cases must be assigned to the small group with changes in the colon only Here, as in most cases in this group, no relevant coexistent intestinal disease was found

DISCUSSION

sp

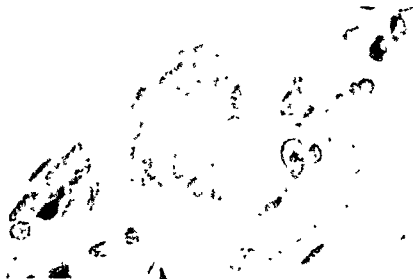


Fig 4
Cyst wall with two multinuclear giant cells × 840

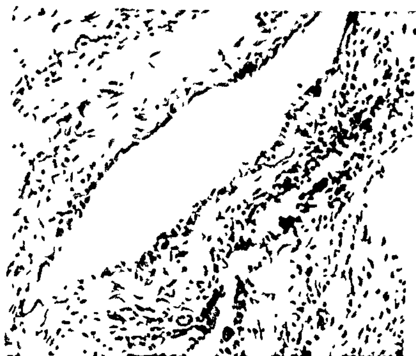


Fig 5
Slit shaped cysts in fibrous tissue with scanty round cell infiltration Partly desquamated epithelium and giant cells in cyst lumen × 83

flexure the subserosa and submucosa contained numerous pinhead sized to walnut sized cysts (Fig 1) Similar cyst formations were also seen in some parts of the large omentum adherent to the changed parts of the colon When compressed with a pincet these cysts burst like small balloons In the part of the colon involved the lumen appeared to be only slightly narrowed The ascending colon the caecal pole the small intestine and the stomach showed nothing of interest Nowhere in the gastro-intestinal tract could obstructions or ulcerations be demonstrated The gall

bladder showed no gross or microscopic signs of acute or chronic disease and the bile ducts contained no concrements

some areas slit shaped cysts were seen and some of them were filled with desquamated endothelium and giant cells (Fig 5) Some of the cysts had no endothelial lining The mesenterium with the lymph nodes jejunum and ileum as well as the stomach showed nothing of interest

Case 2—A 75 year old woman who had had hypertension for 20 years At 57 she had had a spell of allergic rhinitis and examination of the blood on that occasion showed 32 per cent eosinophilic leucocytes and lymphocytosis of 9 300 At 67 years she had pleuropneumonia gastroenteritis and thrombophlebitis From then on she suffered from increasing shortness of breath even on slight exertion and cyanosis and nocturnal attacks of dyspnoea with wheezing respiration Severe pulmonary emphysema and bronchiectasis were diagnosed The respiratory trouble was interpreted as cardiac asthma

increased residual lung

signs of pulmonary alveolar ven

constipation and flatulen

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DISCUSSION

Most authors agree that the cysts represent gas filled changed lymph spaces an opinion supported by the histological picture and by the fact that similar changes are sometimes found in the lymph nodes The cysts

[1] kindly placed at my disposal by Professor *Folke Linell* Path Institute Malmö

are usually situated in the subserosa, but particularly in the caecum, and in the neighbourhood of ulcerations in the intestine, cysts are also seen in the submucosa Some authors have found that the changes are situated mainly in the submucosa in children (cit 3) and mainly in the subserosa *in adults This localization of the cysts in the subserosa or* submucosa may be explained by the anatomical arrangement of the lymph vessels

The slit-shaped cyst spaces may represent old cysts undergoing obliteration with fibrosis in the surroundings The disease has a tendency to heal spontaneously in human beings as well as in animals Later only small granulomas may sometimes be seen, possibly with a few *giant cells These giant cells usually show no phagocytized foreign body* substance Neither was any such phagocytized substance seen in the present cases The inflammatory lesions in the surroundings of the cysts sometimes show eosinophilic lymphocytes and epitheloid cells *Koss* (5) pointed out that demonstration of an endothelial lining of the cysts is necessary to distinguish them from postmortem emphysema or gaseous emphysema during life and not related to the lymph vessels As a rule, the cysts are not in open communication with one another The submucous cysts are usually covered by intact mucosa but necrotic mucosa has been observed This necrosis has been ascribed to impaired blood supply to these parts of the mucosa owing to the cysts (cit 3)

Aetiology

Various theories including bacterial, mechanical-traumatic, physical chemical and alimentary, have been put forward to explain the pathogenesis of the condition

According to the *bacterial* theory, the gas is formed locally by gas *forming bacteria Naeslund had cultured a gas-forming organism from* the cysts of hogs (cit 4) *Kohler* (7) believed to have shown that in one of his cases gas-forming bacteria was the cause of p e i in the rectum Other authors state that in recent years no valid arguments have been presented against the bacterial theory (4) Bacteriological culture, however, usually proves negative (5 and others) A point arguing against this theory is that on rupture of subserous cysts, pneumo-peri*toneum occurs but never peritonitis* (5)

The *mechanical traumatic* theory is the one that is probably most widely accepted According to this theory, the gas is forced, under pressure, from the intestinal lumen through gross and microscopical mucosal defects into the intestinal wall Ulcers with obstruction of the pylorus, which is common in pneumatosis of the small intestine is said to be one of the most important causes *Koss* (5) believes that a more careful study of the appendix would reveal the portal of entry of the gases in cases of primary pneumatosis in the caecal pole But, as mentioned above, in pneumatosis of other parts of the colon and particularly, of the sigmoid flexure, mucosal lesions and associated intestinal

disease appear to be rare (16 19) In this connection it is of interest to note that several authors have recently described cases of pneumatosis in the sigmoid and left colon (4 6 8 9 10 13 15 16 19 20) and relate the condition to some previous sigmoidoscopy (6 9 10) or intestinal operation (20) It might be objected that this theory can explain how the gas enters the intestinal tissue wall but it cannot explain why it is retained there

A much more comprehensive theory the *physical chemical* is based on the absorption of the gases in the intestines Some gases are readily absorbed such as O_2 and CO at which changes in the partial pressure of the gases are of importance for example in fermentative dyspepsia and other alimentary (digestive) disorders with increased production of intestinal gas (1 17) Experimentally it has also been possible to aerate the walls of the intestine by placing an intestinal loop into a gaseous solution (11) Gases are transported from the intestinal tissues mainly in chemically bound form *e g* CO as carbonate and the lymph has proved to have a high CO content (11 17)

In pathological intestinal fermentation with the production of abnormal amounts of lactic acid the intestinal tissues become acid with a possibility of releasing CO for example from the carbonate in the intestinal wall and vessels (11) These acid environments reduce the absorption of gases from the intestinal tissue and this reduction is *increased still* more by the delay of the transport of the gases by a local retardation of the circulation The gas released is then *trapped* in the lymphatics with their slow circulation and *forms cysts* (1) According to some authors (5 and others) the gas can cause secondary tissue *changes* with chronic inflammation and then the gas can be trapped in cystic spaces

The absorption of gas from the intestinal lumen may possibly also increase with *increased* gaseous pressure in the lumen *e g* in abnormal peristalsis because of constipation or attacks of coughing in patients with *asthma In the* presence of constipation peristalsis in some *parts of the* intestine is decreased with reduction of the lymphatic circulation as a result

In addition to the above mentioned local *factors* general factors may also be considered Respiratory *insufficiency on* a cardiac or pulmonary basis for example increases the partial tension of some gases in the blood especially of CO and *thereby in the* tissues and lymph and in addition tends to lower the pH of the blood These fact -
the
deci
tion [illegible] tissues Hence *factors* with a generalized effect presumably contribute to the local causation of *p c i* This applies to *p c i of the* colon as well as of *other parts*

Comment As mentioned no gastroduodenal ulcers or other associated intestinal diseases were found in case 1 in which rectosigmoidoscopy

had not been done either No cultural studies were performed The patient had asthma with emphysema as well as symptoms of general circulatory disorders Postmortem examination showed emphysema and bronchiectasis These clinical and postmortem findings were thus the basis for the above-mentioned systemic factors producing p c i according to the physical-chemical theory One might imagine that the frequent intermittent intra-abdominal variations in pressure due to asthma, might have forced gas into the tissue and lymph spaces, where the gas might have been retained because of impaired absorption In this case the intestinal changes were situated partly in the twisted loops of the colon This has been described previously in several cases and it has been supposed that pneumatosis was secondary to a volvulus *Koss* (5), however, inclines to the view that the gas cysts produce an abnormal peristalsis which in turn gives rise to volvulus

In case 2 there was a cardio-vascular disorder of the general circulation as well as equally severe respiratory insufficiency owing to marked emphysema of the lungs and congestion of the pulmonary circulation The presence of circulatory disorders of the portal system was also indicated by chronic congestive changes in the liver The patient also suffered from flatulence and constipation, suggesting disturbed intestinal fermentation, and she had melanosis of the colon In this case, then, all conditions necessary for the development of p c i, according to the physical-chemical theory, appeared to be satisfied

Roentgen examination of the colon 5 years before death revealed no signs of p c i That examination with retrograde infusion of the contrast *medium might possibly have been a precipitating cause of p c i* in a patient with impaired absorption of gas in accordance with the line of thought set forth above The scar in the stomach might suggest that the ulcer was the portal of entry of the gases, but the region of the pylorus was not obstructed, and obstruction of the pylorus appears to be an important part of the pathogenesis This makes the theory less likely Neither was p c i demonstrable in the small intestine which one might otherwise have expected *Shennon*'s hypothesis that gas can dissect along the mesenterium and cause p c i in various parts of the gastrointestinal tract is, as he himself admits, not well documented (5) No pathogenetic importance can be attached to the bacteriological finding of ordinary coliform rods and enterococci in the intestine

Symptomatology

The symptomatology is not pathognomic In the vast majority of cases of secondary small intestinal pneumatosis the basic disease is often predominant Alternating diarrhoea, constipation and flatulence as well as mucous, blood stained stools have been described Some cases have, however, run an asymptomatic course Large cysts, often conglomerates of cysts have proved to be able to cause intestinal obstruction, which is furthered by the tendency to the formation of ad-

hesions in p.c.i. Volvulus, as mentioned above, and invagination have been reported. Pneumoperitoneum owing to rupture of subserous cysts is not uncommon. In 1 case on record (18) the cystic part of the intestinal wall ruptured.

Fatal sprue-like conditions have also been described as a consequence of widespread p.c.i. (21).

Course

Owing to the vague symptomatology of the disease, it is difficult to date its onset, but evidence is available that relatively advanced changes can occur within a few weeks, but also regress (1, 2, 5) rapidly and in most cases spontaneously. On the other hand cases have been described in which the disease has been followed for several years, sometimes with progression of the changes. In several cases p.c.i. is an incidental finding at postmortem or operation. In other cases, particularly in recent years, the diagnosis has been made on sigmoidoscopy and roentgenographs, when the features of the disease are characteristic (1, 2, 11, 16, 19). In case 1 the diffuse abdominal symptoms and cholecystitic-like pains may be ascribed to the patient's pneumatosis; if so she must have had the condition for one or more years. Owing to the mental state of the patient, however, it was not possible to judge the symptoms with any degree of certainty.

In case 2 the patient had reported constipation and flatulence, which symptoms had probably existed long before the onset of pneumatosis, which probably developed after the age of 71 years, since roentgen examination of the colon on two occasions at that age had revealed no signs of a pathological condition.

Therapy

S

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[illegible] intestinal resection only in the event of obstruction or prolonged distracting symptoms. In primary cases, i.e., particularly pneumatosis of the colon, expectant treatment should be tried first in order to give the disease a chance of healing spontaneously. In every case of pneumatosis, however, the patient should be examined for any other underlying or associated disease. The possible value of correction of circulatory and respiratory disorders and blood gases should perhaps be considered.

SUMMARY

Pneumatosis cystoides intestinalis is briefly discussed, and 2 illustrative cases involving the left colon and the sigmoid, and the entire colon, respectively, are described. The development of p.c.i. in this part of the intestine has been ascribed to traumatization of the colon at

operation or at rectosigmoidoscopy in some cases No such trauma was known in the cases described

In the present cases, pulmonary and circulatory functions were impaired and the significance of this impairment as a cause of p c i is discussed

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FROM INSTITUTT FOR PATOLOGISK ANATOMI UNIVERSITETET I OSLO
(HEAD PROFESSOR OLAV TORGERSEN M D)

THE FREQUENCY OF TEMPORARILY INACTIVE GLOMERULI IN MICE UNDER PHYSIOLOGIC CONDITIONS

By

ODD E. HANSSEN

Received 28 II 61

In 1859 *Hermann* advanced the hypothesis that parts of the excreting renal tissue might be inactive while others were active *Richard & Schmidt* (1922, 1924) provided experimental evidence for this idea as far as the activity of the glomeruli is concerned

Their classical microscopic examinations of the glomerular circulation in living frogs revealed

"The number of glomeruli through which blood flows and hence which function at any one time may be a fraction only of the total number of glomeruli in the kidney This fraction is susceptible of increase by various vaso dilator agencies and of decrea[illegible]

Th[illegible] single glomerulus is variable in anal[illegible]

Ri[illegible] spontaneous intermittence of the glomerular circulation

"A number of glomeruli are watched Flow in one [illegible]

Since then variations in the activity of glomeruli effected either by intermittent circulation in the whole glomerular tuft or in single capillaries have frequently been considered to be involved in physiologic as well as pathophysiologic adjustments of renal function both in amphibia and mammals

The observations of Richard & Schmidt (1922 1924) were soon supported by several investigators while others [illegible]
feature c[illegible]
recently [illegible]
the glom[illegible]
"That [illegible]

[illegible] ust [illegible]
less subtl[illegible]
the physi[illegible]

The que[illegible]
mammals [illegible]
less acces[illegible] of studies In the past however [illegible] the nephron population is [illegible] the problem has been approached by different methods in different species

A method for direct microscopic visualization of the mammalian kidney was devised by *Walker & Oliver* (1941). They were, however, able to observe glomerular circulation in one glomerulus only in each of six guinea pigs out of one hundred examined. In no instance was intermittence observed. These authors and later *Gottshall & Mylle* (1956), employing a similar technique, found a steady and even flow of the proximal tubular urine in rats. This was taken as an argument against intermittent glomerular circulation in these animals. *Hartman* (1955) presented evidence of total and capillary glomerular intermittence in immature mice in which the blood flow of the superficial glomerular tufts were observed by in vivo microscopy. Although species differences may exist, the small number of nephrons observed and the short total time of each observation in the former studies may be responsible for the discrepancy. On the other hand, the functional state of the immature mice used by *Hartman* (1955) may be questioned since it was not controled.

Attempts have also been made to visualize indirectly the circulation of the mammalian kidney by examination at necropsy, of the distribution of certain substances previously injected intravascularly. *Khanolkar* (1922) injected hemoglobin intravenously in rabbits. *Hayman & Starr* (1925) introduced Janus green B into the aorta of laparotomized rabbits. These authors found a varying number of glomeruli free of the substance injected irrespective whether it was allowed to circulate for several minutes or for a few seconds only. *White* (1940), on the other hand, believed that under physiologic conditions the glomeruli of the mammalian kidney were open continuously. He examined the glomerular circulation in rabbits and dogs by injecting Indian ink in the renal artery made accessible by operative intervention. The renal circulation was stopped by clamping the vessels 5 seconds after the injection ended. The histological preparations revealed either that all glomeruli had been filled or, when uninjected glomeruli were observed, their distribution was such as to favour the interpretation that failure of injection was due to uneven distribution of ink in the larger preglomerular vessels rather than to closure of the glomeruli. By use of a principally similar technique *Heggie* (1952) and *Perez-Tamayo & Hernandez Peon* (1953) verified the findings of *White* in rats and cats respectively. These authors, however, observed uninjected capillary loops within the tufts which they believed were indicating glomerular capillary intermittence.

The conclusions drawn from the use of these methods are, however, limited by certain sources of error. Hemoglobin, as used by *Khanolkar* (1922), is known to produce renal injury, and the immediate effect of this substance on the kidney is problematic (see inter al. *Harrison et al.* 1947). The other experiments referred were all made in anesthetized animals subjected to operative intervention with no examination of the renal function. The renal functional state may then have varied and can not be regarded as physiologic without reservations. *Still & Whitcomb* (1954) avoided this error by placing a polyethylene tubing in aorta of rats 3–5 days prior to the final experiments. They were then able to inject Indian ink with minimal risk of excitation of the conscious animals. The renal circulation was stopped by guillotining the rats between the heart and the renal vessels 5 seconds after the injection had begun. By this technique *Still & Whitcomb* (1954) found uninjected glomeruli in 4 of 13 untreated control rats which in the authors opinion raises the possibility that glomerular intermittence may be a normal functional possibility.

As pointed out by *White* (1940), however, the distribution of intraarterially injected substances may vary by chance owing to the possibility of laminar flow and to the effect of skimming within the renal vascular systeme. Moreover, little is known about how far the kidney responds or fails to respond when relatively large amounts of foreign substances are suddenly plunged into the aorta or renal artery.

In order to avoid these objections *Hartman & Flagg* (1950) and *Hartman & Bonfilio* (1959) injected the fluorescent dye vasoflavin in the vena cava of mice, rats and dogs. This route of injection secured an even distribution of the dye in the arterial blood and vasoflavin was considered to be nontoxic. The results of their experiments indicated that in the normal animal all the glomeruli are not patent to flow of plasma at any one time. This, however, applied to anesthetized and laparotomized animals in which no control of urine production was made.

Circulatory activity in the glomeruli of the mammalian kidney has naturally also been studied by clearance methods. Results thus obtained have, however, led to conflicting views with regard to the question of glomerular intermittence. This is

illustrated by studies in rabbits in which the presence or absence of proportionality between glomerular filtration rate and urine flow has been a matter of dispute. A detailed discussion of the topic is given by *Smith* (1951).

Smith et al. (1943), using their well known "glucose titration" method in normal humans, found that the activity of the majority of the glomeruli when defined re- ... whole ... ting as ... ts were

... as artifact with little physiologic significance, and *Dempster et al.* (1956) have questioned the constancy of the maximal tubular reabsorption of glucose.

Another factor which may be considered in "glucose titration" studies is the fact that once the nephrons begin to reject glucose they are subjected to the condition of osmotic diuresis. This probably causes dilatation of the tubules as the author (1960 b) found to be the case ...
sure ...
imme ...

... 350 *Pitesky & Last* ... interpreted the re- ... inactive glomeruli ... the number of which may vary on demand.

So far, the studies referred to do not seem to give an unequivocal answer to the question of the physiologic glomerular activity in the sense of intermittence. Owing to the complexity of factors ...

... ou now as hitherto used can yield conclusive results even when the requirements discussed above are successfully met. As pointed out by *Weawer et al.* (1956) the distribution of injected substances gives only rough information as to the functional vascular morphology since blood readily flows out when the renal vessels are cut. Even, however, when this is prevented by clamping the renal vessels, intravascular displacements may occur as demonstrated by the author in a previous study (1960 b). These post mortem events operate within the time necessary for excision and fixation or examination of the kidney.

Th ...

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tio ... ferrocyanide which is excreted by glomerular filtration. Hence, the problem of glomerular intermittence

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Micro lissection was performe l under a l in ocular dissecti n micrc scope at magni fications of 16 and 25 times The renal surface and the cut surfaces were at first viewed in order to determine the distribution of Prussian blue The tissue to be dis sected was usually taken from the central one third of the kidneys and only occa sionally from other parts By use of transmitted blue light which renders the blue precipitate completely invisible small unselected segments were loosened From about 2 to 5 of these proximal convoluted tubules with their adherent glomeruli were isolated until a number of about 250 was reached The dissected tubules were examined one by one for Prussian blue content by epi illumination of ordinary artificial light against a white background The examination was terminated by a survey of the tissues from different parts of the kidney in search for glomeruli and, or tubules free of precipitate Evidence of total glomerular intermittence with inter vals of at least ten seconds was obtained when the proximal convoluted tubules were found free of visible Prussian blue or a few scattered granules were present only at the tubular outlet from Bowman s capsule

Histological sections which were stained with hematoxylin and eosine were prepared from all the kidneys examined according to the technique used in a previous study (Hanssen 1960 b)

RESULTS

The material and the results of the experiments designed for the present study are listed in Table 1 As seen two of the experiments of Group I were discarded because diffusion of ferrocyanide had occurred during precipitation The one mouse excluded from Group III was regarded irregular because the urine was pink (hemolysis?), and because the intravenous injection of ferrocyanide had been preceded by an unsuccessful attempt During the microdissection of this kidney areas of proximal tubules containing Prussian blue were found alternating with groups of tubules free of precipitate

No histological changes were observed in the sections of the kidneys apart from the occurrence of minute lymphocyte collections preferentially located around larger vessels These were regarded to be of minor significance as no derangement of the renal parenchyma was disclosed The microdissection revealed tubular abnormalities in one mouse of Group IV exhibiting segmental flattening of the epithelium and con striction of the lumen in an appreciable number of the proximal

amounts of precipitate fre-

in this animal seemed re-

periment was discarded In one of the mice in Group III scattered segments of the proximal convolutions were also lined with a flattened epithelium appearing similar to that of the loops of Henle There seemed, however, to be no opstruc tion of the lumen in this case as the ferrocyanide containing filtrate had readily passed as in normal appearing nephrons Therefore this

Prussian

a total of 11403 tubules isolated All the empty nephrons were encountered in 11 of the 40 mice of Groups I–IV, and the great majority of them (98) belonged to one mouse in Group II At microdissection the left kidney of this hydropenic mouse showed alter

is approached by this technique The present study deals with the question of how far glomerular intermittence, with interruption of blood flow of at least 10 seconds duration in entire capillary tufts, may be a mechanism involved in physiologic regulation of renal function Intermittence of shorter intervals or intermittent blood flow in single glomerular capillary loops will be considered in a subsequent paper

MATERIAL AND METHODS

A detailed description of the material and the methods is mainly covered by three previous reports (*Hanssen 1958 1960 a b*) This study comprises 48 adult female *mice weighing between 18 and 25 g*

All animals received 0 05 ml of 10 per cent (occasionally 20 per cent) sodium ferrocyanide freshly dissolved in distilled water A sharp needle was gently inserted in a tail vein In conscious mice one minute or two was allowed to elapse in order to ensure that the mice remained quiet If so the solution was injected in the course of 5 seconds and within another 5 seconds the aorta and vena cava were severed by rapid cutting of the bodies with a pair of scissors

Experiments were performed in five groups of mice subjected to different experimental conditions

Group I represented the condition of the every day life of the mice No restriction was therefore made in the dietary regime ordinarily practiced at our laboratory

Group II In order to obtain the state of maximal concentrating power a small number of mice were fed dry bisquit for three days with no access to fluid The specific gravity of the urine was estimated by the falling drop method of *Jacobsen & Linderstrom-Lang* (1940 41) During the dehydration period the mice lost about 10 to 15 per cent of their body weight, and at the time of injection the specific gravity of the urine was between 1 100 and 1 113

Group III In a third small group the mice were given repeated interperitoneal and subcutaneous *injections of tap water until the specific gravity of the urine* attained values below 1 010 Continuous measurements of urine flow after one intraperitoneal injection of 0 5 ml water was also tried in two mice From preceding values of 0 27 µl/min and 2 4 µl/min diuresis increased to 15 µl/min and 14 µl/min in the course of 17 and 29 minutes respectively At this time the ferrocyanide was injected Before injection is was made certain that the diuresis remained uninfluenced by the vein puncture

All the mice of these groups were kept in holders made of plastic tubing during the experiments The holders had a transverse slit so placed that the bodies could be severed at the desired level without preceeding handling of the animals Immediately after sacrifice the upper half of the tube was removed The left kidney was excised and the whole organ was immersed in a mixture of acetone and solid carbon dioxide This procedure was ordinarily completed in less than 25 seconds

Group IV In this series the mice were anesthetized with nembutal and kept in a *prone position with the extremities stretched The left kidney* was exteriorized and irrigated with a modified Ringer gelatine solution of body temperature The animals were gently heated to maintain a body temperature of about 37° C Flow of urine was measured No injection of ferrocyanide was made before urine flow was at least 2 µl per 5 minutes and the surface of the exteriorized kidney revealed a bright red colour Within an interval of 2 seconds after sacrifice the *left kidney* was pinched off by tying a ligature which previously had been placed loosely around the renal pedicle The kidneys were then transferred immediately to the freezing mixture

Group V In an additional series of 4 conscious mice vein puncture was made in the usual manner Then in order to excite the mice cotton soaked with ether was hold near the animal s nose When the mice appeared disturbed but before any anesthetic effect was notable the ferrocyanide solution was injected

The ferrocyanide contained in the tubular lumina was precipitated according to *the method previously described (Hanssen 1958)* The frozen tissue was divided in suitable pieces and was treated with concentrated alcoholic solution of ferric chloride at —32° C to —34° C for not more than 20 hours

nating areas comprising well filled and more or less empty proximal convolutions Of 102 nephrons isolated from an "empty" field only 4 contained Prussian blue whereas precipitate was seen in all of 92 tubules isolated from a "well filled" area The two areas apparently corresponded to different fields of supply of interlobular arteries The occurrence of numerous glomeruli which had been inactive in the period between injection and sacrifice, could not be related to any technical failure or to pathological changes The other 14 empty proximal convoluted tubules were nearly equally distributed among all the groups

TABLE 2
The Percentage Frequency of Inactive Glomeruli in the Mice of Groups I IV Estimated by the 99 per cent Confidence Intervals for n = 250

Frequency of inactive glomeruli	in	number of mice
< 2 %		29
< 3 %		8
< 4 %		1
< 6 %		1
32 48 %		1

Presuming that the tubules were isolated at random the probable frequency of empty nephrons may be given by the confidence intervals as illustrated in Table 2 When ignoring them, the one divergent animal of Group II (see below) the results indicate that glomerular inactivity in the sense of a cessation of filtration and hence of blood flow of at least 10 seconds duration is not a common feature under conditions of every day life of the mice Neither did extreme dehydration, hydration or operative intervention as employed seem to have had any demonstrable influence upon the number of inactive glomeruli observed It should also be emphasized that the two doses of ferrocyanide did not provoke any different response

A stressing stimulus may, *on the other hand, render the* glomeruli inactive for a period of at least 10 seconds as indicated by the results obtained in Group V As indicated in Table 1 the ferrocyanide excretion showed a widely varying pattern This, however, seems not peculiar since the stressing stimuli may have varied in intensity, and since injection of ferrocyanide can hardly be performed in the same phase of *the stress reaction*

DISCUSSION

The present study is based on the assumption that Prussian blue observed in the tubules represents ferrocyanide which was excreted by *glomerular filtration* Since the ferrocyanide solution was injected intravenously the solute was moreover regarded, as having been evenly

TABLE 1

Experimental conditions	Number of mice: Solution injected 10 %	Number of mice: Solution injected 20 %	Number of mice: Discarded	Number of prox conv tubules isolated	Empty tubules observed: total no	Empty tubules observed: in no of mice	Comments
Group I Mice with no intervention	12		2	4048	6	5	5 of the empty tubules were juxtamedullar
		3	0	613	0		No empty tubules found among 439 dissected *from poles of the kidney* in 2 mice
Group II Dehydrated mice	5		0	1237	98	1	The empty tubules were evenly distributed
Group III Hydrated mice	7		1	1324	6	3	4 empty tubules were found in one mouse and were from outer cortex
Group IV Anesthetized mice with left kidney exteriorized	11		1	3396	2	2	The ext kidney blanched after injec in 2 mice The empty tubules were juxtamedullar
		6	0	805	0		
Total	35	9	4	11403	112	11	
Group V Mice subjected to a stressing stimulus							
	1			0			The ferrocyanide had stopped in the arcuate arteries
	1			139	6		Great differences in the amount of precipitate within the tubules
	1			14	14		A large number of empty tubules evenly distributed throughout the kidney
	1			68	0		No empty tubules observed

The reason for excluding 4 experiments see text

ferrocyanide than glomeruli fully functioning all of the time from commencement of injection to sacrifice Examination of the distribution of ferrocyanide among nephrons (to be referred to in a subsequent paper) lends no support to the idea of such an activating effect of the injected solution

Neither does the restrained position in which the conscious mice were kept under the experiments, nor the vein puncture, seem to be important in this respect since a reverse effect, if any, appears more conceivably mediated by fright or excercise Thus *Bing & Knudsen* (1954) demonstrated that fright may elicit renal vasoconstriction in mice Excercise per se is also known to reduce renal blood flow and glomerular filtration rate (*Smith* 1951 p 438) In accordance therewith, the results of Group V of the present study demonstrate that cessation of glomerular filtration also may be provoked in a varying number of nephrons by stressing stimuli In view of these findings the divergent result obtained in the one hydropenic mouse may be considered to reflect a reaction to fright or struggling In fact, the dehydrated mice were restless and difficult to handle so that it seems rather peculiar that not all of them responded similarly

A transitory vasoconstriction followed by a reactive increment in the number of active glomeruli might still be considered In the author s experience, however, there seems to be no supporting evidence of such a sequence of reactions On the contrary, measurements of the diuretic response to mannitol and ferrocyanide made on conscious mice (*Hanssen* 1960 a) indicated that mice tolerated well both the restrained position and the vein puncture Moreover, a rather heavy stressing stimulus appeared necessary to provoke vasoconstriction Neither seemed an activating effect of the procedure likely to occur in the operated animals As previously shown (*Hanssen* 1960 a) the intervention probably was followed by some reduction of the glomerular filtration rate

The use of the confidence intervals for estimation of the probable frequency of inactive glomeruli necessitates some reservations since sampling of proximal tubules was not made strictly at random However the method used excluded completely any subjective guidance in the selection of tubules showing blue precipitate in the [illegible]

[illegible]

inactive nep[illegible]

the larger r[illegible] local distribution of empty tubules Neither was such a distribution observed at the final inspection by which groups of inactive nephrons were easily discernible in the stressed animals The proximal convoluted tubules of the juxtamedullar nephrons were far more difficult to isolate than the remainder For this reason a relative underestimation of these nephrons can not be excluded With this exception, however, the sampling was considered

distributed in the arterial blood reaching the kidneys Proximal convoluted tubules free of *precipitate were therefore considered as indicating* glomerular inactivity in sense of a cessation of filtration during the period of ten seconds which corresponds to the interval between commencement of injection and sacrifice

Two facts compel a reservation with respect to the term inactivity as used *in this study Tubules found to be empty, i e* free of precipitate, can not be regarded as completely free of excreted ferrocyanide since the sensivity of the Prussian blue reaction in tissues is not exactly known (*Gersh & Stiegelitz* 1934) Moreover, since intrarenal blood may move postmortally (*Hanssen* 1960 b) nephrons had to be considered as *inactive even when the glomerular* capillary tufts stained faintly blue and scattered granules of precipitate were present in the capsular space However, glomerular filtrate flowing into the proximal convolutions could hardly *have been produced to any significant* extent post mortally as the mode of sacrifice used secured an almost instant cessation of the renal circulation If, after all, glomeruli of nephrons thus registered as inactive were in operation during the ten seconds period their filtration rate must have been neglibly small as compared with that of nephrons regarded as functioning In the latter, the amount of precipitate certainly exceeded many fold the lower limit of detection of the substance, and the ferrocyanide-containing filtrate had passed an appreciable distance along the proximal convoluted tubules

The characteristic findings indicate that the majority of the glomeruli *were functioning even under conditions which might be expected* to alter the number of inactive glomeruli Were this remarkable uniformity a consequence of the experimental procedure, untoward reactions must have been elicited which were able to restore the filtration of inactive glomeruli

The most crucial point, so far, seems to be the possibility that the injected solution may have elicited vasodilatation This question could hardly be made the subject of direct control so it thus may be open to criticism *The results were, however, similar for both doses of ferro*cyanide used Evidence is also at hand suggesting that injection of the *solution is more likely to provoke vasoconstriction rather* than dilatation as indicated by the two experiments of Group IV In these cases however, the number of inactive glomeruli were not increased probably because the reaction was delayed This was supported by the fact that the reaction was not apparant until just before sacrifice On the other hand, vasoconstriction seemed to be an uncommon response since injection of 20 per cent ferrocyanide solution ordinarily was followed by profuse diuresis *(Hanssen 1960 a) If, on the contrary an opening up* of inactive glomeruli were a regular response to injection, it seems justified to suggest that this reaction, like vasoconstriction, would have *needed* a certain period of time to develop This involves the conclusion that glomeruli thus activated would have excreted a smaller amount of

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to be representative for the nephron population of at least the central third of the kidney The results also seem valid for other areas as verified by dissection of tubules from the poles in two experiments Moreover, inspection of the different areas never disclosed a larger number of inactive glomeruli than present in the central part, and in the stressed animals the occurrence of empty tubules was found to be evenly distributed throughout the kidneys

SUMMARY

The frequency of inactive glomeruli in the sense of a cessation of filtration of at least 10 seconds duration was investigated in mice by a method described by the author (1958) Sodium ferrocyanide was used as an indicator of filtration Following intravenous injection of this substance the excretion was visualized by precipitation as Prussian blue Registration was made after microdissection of the proximal convoluted tubules and their attached glomeruli Glomerular inactivity, and hence cessation of blood flow, was considered to exist when no blue precipitate was discernible in the proximal convolutions This technique is believed to meet some of the objections which can be made on certain earlier studies on the topic

The results revealed a very low frequency, probably less than three per cent, of inactive glomeruli in mice during the conditions of their everyday life This frequency seemed moreover not to be altered neither by excessive hydration or dehydration or by anesthesia combined with exteriorization of one kidney This leads to the conclusion that intermittent glomerular blood flow over intervals of at least ten seconds is not a likely mechanism of physiologic regulation of renal function in mice

The majority of the few inactive glomeruli found belonged to the juxtamedullary nephrons This observation may raise the question as to whether glomerular intermittence in the juxtamedullary nephrons can be a mechanism for regulation of blood flow through the medulla The observations on this anatomical group of nephrons are, however, too few so that the problem, at the present time, must be left open

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FROM INSTITUTT FOR PATOLOGISK ANATOMI, UNIVERSITETET I OSLO
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THE RELATIONSHIP BETWEEN GLOMERULAR FILTRATION AND LENGTH OF THE PROXIMAL CONVOLUTED TUBULES IN MICE

By

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Although the nephrons of the normal mammalian kidney have in [illegible]

urine production a fluid equal in composition and in the amount delivered per unit of time. In recent years, however, more attention has been paid to the problem of what functional significance structural variety among the nephrons may connote.

Oliver (1955) has discussed the validity of renal concepts in view of such anatomic diversities, especially with reference to intrarenal handling of glucose and, on the basis of statistical measurement, outlined a procedure by which [illegible]

[illegible] speak strongly for a multiplicity of nephron activity that must be taken into account in the evaluation of any change in urine formation.

One major problem involved is the understanding of the function in the single glomerulus and its regulation. Thus the volume of filtrate may condition tubular functions (*Bradley* 1957, p. 521), and renal perfusion is dependent on the blood flow through the glomeruli. [illegible]

[illegible] reliable for precise evaluation of the problem in question.

Hence, in the present study, the problem was approached by a new method (*Hanssen* 1958) which allows visualization of filtered ferrocyanide as Prussian blue in nephrons isolated by microdissection. This method has the advantage that the amount of substance indicating glomerular filtration may be compared directly between the various nephrons and can easily be correlated with their size.

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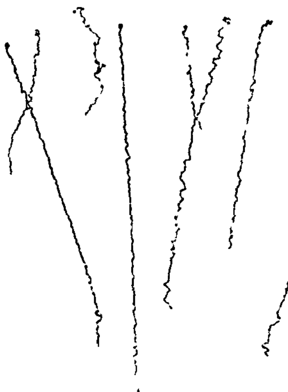


Fig 1
Photomicrograph of proximal convoluted tubules isolated from the left kidney of mouse no 158/57 illustrating the length variations The central tubule is a typical juxtamedullary one the nearest tubule on either side of it is isolated from outer cortex × 14

RESULTS

The present microdissection confirmed the earlier observations (*Sperber* 1944) in that proximal convoluted tubules and the respective glomeruli of mice vary considerably in size (Fig 1) As also known, the various size categories of the proximal convoluted tubules, in these animals, revealed a systematic distribution The shortest tubules were always found in the outer cortex, as their origin approached the inner zone of cortex, tubular length increased and the longest tubules belonged to the juxtamedullary glomeruli The latter often formed long convolutions running parallel to the cortico medullary junction

It was evident that the quantity of Prussian blue present within the tubules also varied Thus as far as could be judged by microscopic examination the amount of precipitate per unit of tubular length (Fig

The present study also deals with the question of how far intermittent glomerular function of very short intervals may be involved in regulation of glomerular function

MATERIAL AND METHODS

The distribution of excreted ferrocyanide was recorded in 1257 proximal convoluted tubules isolated by microdissection These were obtained from 14 of the mice previously investigated (*Hanssen* 1961)

The material was selected for the present purpose from a larger group of animals in which the ferrocyanide was confined to the proximal convoluted tubules only

Half of the animals had been subjected to nembutal anesthesia and to exteriorization of the left kidney In these mice the flow of urine was measured by means of an indwelling bladder catheter (for technical details see *Hanssen* 1960 a) In the other mice no intervention was made All the animals had free access to food until they were used The animals were sacrificed ten seconds after having received 0 05 ml 10 per cent sodium ferrocyanide solution intravenously, by rapid cutting of the aorta and the vena cava between the heart and the renal vessels The time was reckoned from commencement of injection The left kidney of the unoperated mice was excised within 25 seconds, the exteriorized kidney of the operated animals at the instant of killing The excised kidneys were immediately frozen in a mixture of solid carbon dioxide and acetone The ferrocyanide present in the tubules was visualized as Prussian blue by the method previously described (*Hanssen* 1958) by precipitation of the substance with ferric chloride at —32° C to —34° C After subsequent maceration in 20 per cent hydrochloric acid a number of proximal convoluted tubules with their glomeruli were isolated by microdissection In order to avoid subjective selection of tubules according to their content of blue precipitate the dissection was performed in transmitted blue light A number of isolated tubules was mounted on slides and drawn at a magnification of 113 The entire length from the glomerulus to the junction with the thin segment of the loop of Henle including the Prussian blue containing part, were measured by a curvemeter The outline of the blue stained glomerular tufts were drawn at a magnification of 500 The "equatorial" area thus obtained was measured by planimeter

Several untoward factors might be expected to have influenced the measurement of the various dimensions For example the extent of precipitate varied from animal to animal even in tubules of equal length This might be ascribed to variations in glomerular filtration rate among the mice and to the fact that the rate of injection of ferrocyanide and the time of sacrifice were not exactly duplicable Delay in freezing of the kidneys, which was necessary in the conscious mice had been found to produce some postmortal displacement of tubular urine occurring concomitantly with drainage of blood and reabsorption of tubular urine (*Hanssen* 1960 b) The dimensions measured were also influenced by the degree of swelling of the tissue For these reasons the measured dimensions of each nephron were expressed as a ratio between the measured values and those of the smallest nephrons found in the same kidney By this method of procedure the distribution of precipitate and its relationship to nephron size could be compared in different animals

The proximal convoluted tubules measuring 3000 3999 microns were regarded as the smallest and the average of their measured dimensions were used for calculation of the ratios The majority of the shortest tubules found in the outer cortex actually fell within this group Still shorter tubules were occasionally encountered but they were not considered since they were few in number In one mouse however an appreciable number of proximal convoluted tubules measured only 2000 2999 microns The mean of these were used for calculation since in this particular case the renal tissue appeared less swollen after maceration than the parenchyma of the other kidneys investigated The variability of the extent of Prussian blue and glomerular size within the shortest nephrons are illustrated by the standard deviations computed for the total number of both experimental groups (Table 1)

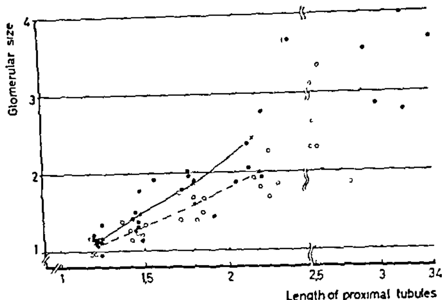


Fig 3 Black and broken lines indicate the average of all observations in unoperated (●) and operated (○) mice respectively

Figs 3 and 4
Relationship between length of the proximal convoluted tubules and glomerular size (Fig 3) and between tubular length and the length of the ferrocyanide column (Fig 4) The figures represent measured dimensions divided by the average values for the smallest nephrons of the same kidney Dots on the left part of the graphs give the mean values for tubules within different length groups of each mouse whereas dots on the right part and in bracket represent single nephrons

Relationship between Glomerular Size and Length of Proximal Convoluted Tubules

Fig 3 points to the existence of a clear correlation between the glomerular area measured and the length of the proximal convoluted tubules Thus, for tubules being *e g* about 2 to 2 5 times as long as the shortest ones, the glomerular area showed an 1 7 to 2 4 fold increment This relationship which appeared almost linear, seemed also to be present even for the longest tubules as indicated by the single observations presented on the right part of the chart The variability of glomerular size (Table 1) among proximal convoluted tubules of nearly equal length certainly exceeded the anticipated ± 10 per cent range within which the tubular length varied This, however, is readily accounted for by the several sources of error involved in these measurements Firstly, the consistence of the tissue after maceration, and the pressure effected by the coverslip during mounting might have altered the size of the "equatorial" area of the glomerular tufts Moreover, drawing of the outlines of the tufts, and the planimetric measurements were like

2) as well as the extent of it was ordinarily found to increase with increasing length of the proximal convoluted tubules. On the other hand among nephrons from the same level in cortex no difference was discernible. This at least was true for tubules being until 2.5 times as long as the "reference" tubules. In longer nephrons, however, greater variations were evident.



Fig. 2
Photomicrograph of the proximal part of a juxtamedullary nephron (a) and a nephron from outer cortex (b) from the same kidney as Fig. 1 showing a marked difference in tubular luminar diameter and in amount of precipitate contained in the tubules. × 240.

The length of the precipitate containing part of the tubules was measured as an approximation to quantitative estimation of the precipitate. In order then to examine the interrelationship between length of the proximal convoluted tubules, glomerular size and distribution of precipitate, the nephrons were grouped according to the ratio giving the relative length of the tubulus. The size categories were arranged as consecutive groups with a range of ± 10 per cent of the arbitrary group means. The reason for this was to keep as far as possible, the effect of the errors not eliminated by the method of procedure within an equal range irrespective of great variations of tubular length. For each kidney, the mean of the relative extent of precipitate and of glomerular size within the different nephron groups were computed.

TABLE 1
The Average Length of Ferrocyanide Column Respective Size of Glomerular Equatorial Area within the Different Length Groups of the Isolated Tubules

Range and class mark ( ) of the tubular length groups	Operated mice			Unoperated mice		
	No of ols	Length of ferrocyanide column	Size of glom equatorial area	No of ols	Length of ferrocyanide column	Size of glom equatorial area
		Mean ± C	Mean ± C		Mean ± C	Mean ± C
Shortest tubules						
0 900 1 099 (1 000)	85	1 000±10 9	1 000±14 0	278	1 000±15 9	1 000±21 7
1 100–1 344 (1 222)	207	1 143±11 3	1 075±15 7	264	1 181±16 4	1 151±21 4
1.345–1 643 (1 494)	100	1 301+12 4	1 270±18 5	155	1.388±23 4	1 437±38 8
1 644–2 008 (1 826)	44	1 527+11 7	1 595±20 2	59	1 728±22 1	1 859±30 0
2 009 2 445 (2 227)	22	1 712±15 2	1 997±20 1	30	1 920±31.8	2 444±34.3

The figures represent the measured dimensions expressed as their ratio to those of the shortest nephrons of the same kidney C standard deviation in per cent of the mean

Relationship between Extent of Precipitate and Length of Proximal Convoluted Tubules

A correlation between the extent of precipitate and the length of the proximal convoluted tubules was also demonstrated (Fig 4)

In the operated mice this correlation seemed to be nearly linear for nephrons with a length of proximal convoluted tubules varying within a 2 5 fold range, but the correlation was apparently not extended to longer tubules as indicated by the single observations on the right part of the graph In unanesthetized mice, the relationship in question was not evident In three of them it was valid for proximal convoluted tubules up to about 2 5 times as long as the average of the shortest tubules In three other mice, however, the extent of Prussian blue did not increase to the same degree with increasing tubular length (no tubules with length ratios exceeding 1.5 had been mounted in the seventh untreated mouse) As discussed below, the discrepancy may, however, be ascribed to experimental artifacts

The variability in distribution of Prussian blue among tubules of equal length is illustrated by the histograms presented in Fig 5 and by Table 1 The class intervals of the histograms were given a range of ± 10 per cent of the arbitrary class marks, corresponding thus with the respective length groups As seen, far the greater majority of Prussian blue ratios were distributed in three neighbouring class intervals in all length groups below 2 5 When allowance is made for the variability

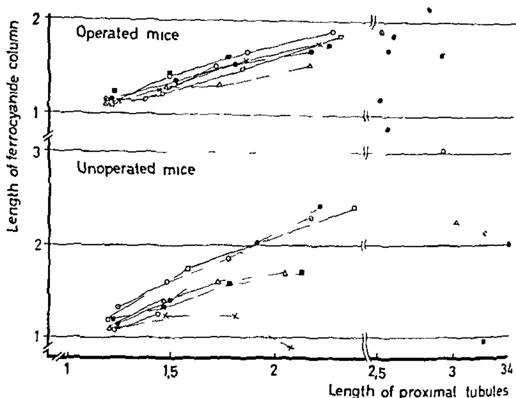


Fig 4 Identical symbols denote tubules from the same kidney

wise liable to certain inaccuracy The possibility of different degrees of stretching of the nephrons is regarded to be the most important source of error since it would provide risk for an erroneous grouping of the respective tubules

The fact that the size of glomeruli belonging to proximal convoluted tubules of nearly equal length varied more in unanesthetized than in operated mice (Table 1) is considered attributable to postmortal events It has been shown that blood drains rapidly from the renal vessels when the renal pedicle is cut, and this occurs at unequal rates in different areas (*Hanssen* 1960 b) For this reason the blood content of the glomerular tufts probably was different in the unanesthetized mice at the time of freezing their kidneys involving certainly also the size of the tufts

The somewhat peculiar observation that the relative glomerular size, on the average, appeared lower in the operated animals than in the untreated mice (Fig 3) seems also attributable to early post mortem changes In general, the vessels of the outer cortex apparently emptied first (*Hanssen* 1960 b) which then would give too high relative values for glomeruli situated deeper in the cortex

mice (Table 1 Fig 5) The majority of Prussian blue ratios (80 to 90 per cent) were distributed in three neighbouring classes as in the former group In the remaining nephrons however, the ratios were widely distributed among the lower class intervals These tubules were mainly found in the three mice which showed somewhat divergent behaviour and in which the relationship between the extent of precipitate and tubular length seemed to approach that found in operated animals (Fig 4) The reason for this may again be found in the events shown to occur postmortally (*Hanssen* 1960 b) Once the renal circulation is cut off tubular urine is reabsorbed Concomitantly some distal displacement of the tubular content takes place probably at unequal rates in different tubules as indicated by the changes observed in the luminar diameter early post mortem A slight transitory vasoconstriction elicited by the procedure of injection might also be considered responsible for the variability of the extent of precipitate The fact that only a small percentage of nephrons mainly in half of the mice were involved points to the latter possibility Thus a stress stimulus was found to provoke a highly variable response (*Hanssen* 1961) Irrespective of which of these factors had been the most decisive the heterogeneity under discussion however seems fully attributable to experimental artifacts rather than to real functional disparities

As far as the single observations are concerned the extent of precipitate showed considerable variations in the longest proximal convoluted tubules This feature may probably not have been caused by early post mortem changes since it was present in both experimental groups Neither can different degrees of stretching of the few tubules available have been a decisive factor since it would hardly have changed the proportion between extent of precipitate and tubular length to any significant degree

In the untreated mice the extent of Prussian blue and the length of proximal convoluted tubules in general were related in a proportion of 1 to 1 (Fig 5) except in the longest tubules (Fig 4) where the proportion was below 1 The other experimental group showed a less increase in the extent of precipitate at increasing tubular length (Figs 4 and 5) According to earlier observations (*Hanssen* 1960 b) the post mortem displacement of tubular content occurs latest in the deep nephrons In the untreated mice this would tend to give low ratios for the extent of precipitate in these longer tubules Hence the difference between the experimental groups were regarded real

DISCUSSION

With reservations made for some probably insignificant limitations (*Hanssen* 1958) the total amount of precipitate present in the proximal convoluted tubules was considered to represent the quantity of ferrocyanide filtered by the glomeruli in the interval between injection and

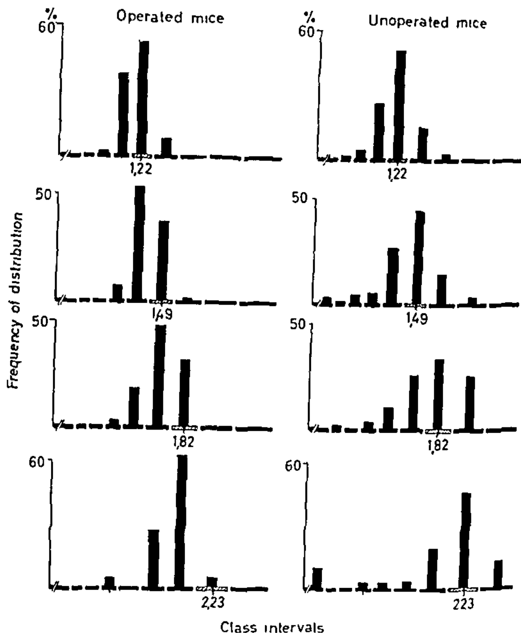


Fig 5
Histogram showing the frequency distribution of the length of the ferrocyanide columns within the tubular length groups in both experimental series The class intervals were given a range of ± 10 per cent of the class marks as for the length groups The number of observations within, and the range of the length groups are given in Table 1 The group which each histogram represents is indicated by the class mark

anticipated by the arbitrary range of the length categories, and for methodological errors, the results indicated a fairly even distribution of precipitate among tubules of equal size in the operated mice

The distribution of precipitate was not, however, found to be so uniform among equal proximal convoluted tubules of the untreated

disregarding the methodological errors, and the fact that the tufts not always appeared as spheres, the volume of the tufts may roughly be deduced from the area measured. Thus, 3 to 4 fold increase in this implies between about 5 to 8 fold increase in volume.

Hence, as far as mice are concerned, the present results fail to support the hitherto accepted assumption of equal glomerular filtration rate for all nephrons. This inequality appeared to depend upon the anatomic heterogeneity demonstrated.

Among anatomically identical nephrons, however, glomerular filtration rates seemed equal. This was evidenced by the remarkable uniformity of the extent of precipitate and of the amount of precipitate present per unit of length of the proximal convoluted tubules. The somewhat divergent behaviour of conscious mice probably was attributable to methodological artifacts. The possibility that ferrocyanide per se should have an effect tending to level out disparities in glomerular filtration within the short intervals seems untenable. The results are therefore considered to be a strong argument against any significant occurrence of intermittent glomerular blood flow either in whole capillary tufts or in parts of them, even for very short time intervals.

For these reasons, and due also to the fact that glomerular filtration and glomerular size seemed closely related, the varying glomerular filtration found among the nephrons is regarded as being a consequence of corresponding variations in the capillary area available for filtration.

It is not likely that the functional and structural diversities are unique features in mice. Anatomic studies (*Peter* 1909, *Huber* 1917, *Pai* 1935, *Sperber* 1944, *Oliver* 1955, *Oliver & MacDowell* 1961) have

These aspects have relevance to the problem concerning the relationship between glomerular filtration of any one substance, and hence the tubular load, and the capacity of the proximal convoluted tubules to process the tubular urine. The "glucose titration" experiments of Smith *et al* (1943

referred to by Smith as "glomerular activity".) Recently *Oliver & MacDowell* (1961) measured the various dimensions of 104 proximal convoluted tubules and their attached glomeruli isolated from 3 human kidneys. These authors found a clear correlation between the surface area of the glomeruli and the volume of the proximal convoluted tubules. Using these dimensions as a "structural equivalent" of filtration and maximal glucose reabsorptive capacity respectively, they were able to derive a hypothetical "glucose titration" curve which matched remarkably well that given by *Smith et al* (1943). A similar conformity was obtained by examination of 138 nephrons iso

sacrifice of the animals Microscopic evaluation of the individual nephrons revealed that the Prussian blue content of the tubules increased corresponding to their length, thus suggesting a similar variability of glomerular filtration A rough approximation as to the range within which the glomerular filtration rate might have varied was reached by measuring the extent of precipitate Because ferrocyanide, in all probability, was evenly mixed with the renal arterial blood, it may be assumed that the concentration of the substance was equal in the filtrate of all glomeruli when produced at the same time Then, at a given glomerular filtration rate, the extent of the precipitate must be a function of the luminar volume Although the diameter of the proximal convoluted tubules was not regularly measured, it was evident that the diameter, within certain limits, increased with increasing tubular length, as has been described by *Sperber* (1944) This was also supported by the fact that the amount of precipitate per unit of length appeared greater the longer the tubulus when the microscopic comparison was made in corresponding parts of the tubules The length of the precipitate-containing part in the deep proximal convoluted tubules was frequently more than twice that of the shortest nephrons from the outer cortex, as was also the diameter (*Hanssen* 1960 b) Consequently the results suggest that the glomerular filtration rate had varied at least within an eight fold range

This observation is tenable only in so far as the effect of other factors upon the distribution of the precipitate can be excluded Thus, a similar variation in the extent of Prussian blue might have been obtained with even glomerular filtration rate throughout, if proximal water reabsorption had varied inversely proportional to the tubular length There seems, however, to be no reason why longer tubules, certainly having a greater mass, should have a less water reabsorption than shorter ones Were this the case, the amount of precipitate per unit of tubular volume should be less the longer the tubules The results provided no evidence for such an idea Moreover, the relation apparently existing between nephron size and glomerular filtration rate argues against the possibility that the difference in time at which ferrocyanide reached the glomeruli might have been responsible for the varying extent of precipitate The effect of post mortem events on the distribution of precipitate could not be fully eliminated but these might not have been able to elicit the principle pattern of distribution In fact, this factor might rather work in the opposite direction Lastly, the variability of the activity of the glomeruli was found to be a common feature in all the mice investigated

The functional observations seemed to agree well with the anatomical data showing a close correlation between glomerular size and filtration The glomerular filtration rate should be a function of the capillary length which probably is related to the volume of the tufts rather than to the "equatorial" area as measured in this study When, however,

cortex to the tip of the medulla with a nearly equal osmolarity in adjacent structures at any level along the longitudinal axis. The efficiency of such a system increases with increasing length of the hairpin loops. The present results indicate that the amount of filtrate produced in any single nephron of the mouse kidney varies corresponding to how far the loops of Henle penetrate the medulla and is greatest in the juxtamedullary nephrons whose loops run nearly to the tip. This implies that the longer the loops, the greater is the amount of solute carried to the site of reabsorption. This may contribute to the efficiency of the countercurrent mechanism in animals with a nephron population of the mouse type, whether it operates as a multiplier system (Wirz 1957, *Gottschalk & Mylle* 1959) or as an exchanger system (*Berliner et al.* 1958).

Wirz (1953) and *Gottschalk & Mylle* (1959) have demonstrated experimentally that vasa recta participate in the countercurrent system making it more efficient, probably by trapping solutes and carrying away water. *Berliner et al.* (1958) have particularly stressed the importance of the medullary blood flow and have pointed to the fact that minor changes may have a major effect on urinary concentration. Some recent experimental evidence actually support this idea. *Lilienfield et al.* (1960) estimated the medullary plasma flow in dogs by measuring the accumulation of I^{131} labeled albumin. On the basis of their data the authors suggested that the plasma perfusion rate of the renal papilla varies with induced alternations in solute and water relationships and that hitherto unsuspected vascular mechanisms may be operating.

The present experiments demonstrated that the glomerular filtration rates of the juxtamedullary nephrons varied and hence probably the blood flow through vasa recta really may be variable. Thus the difference in tubular content of ferrocyanide among these anatomic group of nephrons seemed to be greater than what may be accounted for by size differences and possible errors. Even though the size of the glomeruli revealed great variations, measurement of these was considered liable to greater inaccuracy than measurements of the extent of precipitate. Although the observations were rather few, they are thought to favor the concept [illegible]

[illegible] ould in the juxtamedullary zone. The distributional changes in glomerular filtration rates between deeper and outer nephrons might suggest another possible regulating mechanism. However, no attempt was made to relate these findings quantitatively to the functional state of the mice due in part to limitations of the measurements of precipitate and because the experimental conditions could not be controlled exactly. On the other hand, the experimental results seem sufficiently suggestive to merit further studies of some

lated from 3 dogs in which "titration" experiments had been performed (*Bradley, Laragh, Wheeler, MacDowell & Oliver* 1961)

The present study showed, in general, a correlation between glomerular filtration and the length and probably then the mass of the proximal convoluted tubules In fact, the results obtained in mice might be regarded as a direct demonstration of the validity of certain theoretical assumptions which the authors referred above had to consider when they deduced the tubulo-glomerular relationship ("glomerular activity") from the structural and functional measurements made in man and dog

The present results, however, revealed that the relationship between *the amount of ferrocyanide excreted and tubular length found in the* great majority of nephrons deviated in the deepest ones This may be compatible with the fact that the frequency distribution diagrams presented by *Smith et al* (1943), *Oliver & MacDowell* (1961) and *Bradley et al* (1961) show a small skew indicating a deviation of "glomerular activity" in a small group of nephrons It should also be emphasized that the "functional-structural" relationship seemed to be influenced by the experimental conditions in the mice

The extensive comparative study of *Sperber* (1944) revealed the anatomic pattern of nephron population to be of the "mouse type" in several mammals, but primarily in the smaller ones *Bowman* (1842) and *Pai (1935) claimed that the deepest glomeruli in human kidneys were larger* than those from outer cortex *Truela et al* (1947) were unable to confirm this *Oliver & MacDowell* (1961 and in *Bradley et al* 1961) who dissected a rather large number of proximal convoluted tubules with attached glomeruli from human and dog kidneys, found the nephron population randomly distributed with respect to size

Functionally the ability of concentrating the urine varies among the species Mice may elaborate a more concentrated urine than rats (*Hanssen* 1960 a) whose concentrating power is greater than that of dog and man in that order (*Corcoran et al* 1956) It has repeatedly been emphasized that the ability to produce urine hypertonic to plasma depends upon the presence of thin segments *in the loops of Henle, and* that the concentrating power is positively correlated with the length of the loops (*Peter* 1909, *Sperber* 1944, *Schmidt-Nielsen* 1958)

In 1951 *Hargitay & Kuhn* proposed a new theory concerning the urinary concentrating mechanism, according to which the loops of Henle play a critical role in production of hypertonic urine In recent years, a considerable amount of experimental evidence has accumulated in favour of their concept although the true nature of some of the events involved still are debatable (see *a o Wirz et al* 1951, *Wirz* 1953, 1957, *Berliner et al* 1958, *Gottschalk & Mylle* 1959, *Lamdin* 1959, *Ullrich* 1959, *Leaf* 1960) In essence, the hypothesis presumes reabsorption of solutes, *mainly sodium and chloride*, by some unknown process in the loop of Henle By the effect of countercurrent flow through the hairpin-like loops, an increasing concentration of solutes is built up from the

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of the aspects discussed It is believed that the present method should be of value in this research work when quantitative estimation of the precipitate can be performed in tubules from animals in which the experimental conditions have been more precisely controlled

SUMMARY

The glomerular filtration was investigated in the nephron population of mouse kidney and correlated with the size of the glomeruli and with the length of the proximal convoluted tubules This was done by the precipitation of excreted ferrocyanide as Prussian blue and evaluation of the precipitate in proximal convoluted tubules isolated by micro dissection

The results indicated a steadily increasing glomerular filtration in the nephrons as their origin approached the cortico-medullary border The glomerular filtration rate seemed to be closely related to glomerular size and to the length of the proximal convoluted tubules It was determined that the glomerular filtration rate of the juxtamedullary nephrons approximates eight times that of nephrons from the outer cortex

Among nephrons of equal size lying in outer cortex, the glomerular filtration appeared fairly uniform This argues against the assumption of intermittent glomerular blood flow even for very short time intervals In contrast, in the nephrons of inner cortex the amount of precipitate varied more than might be attributable to technical variations It was further demonstrated that the relationship between filtration rates of the outer and deeper nephrons was influenced by the experimental conditions

The anatomic and functional heterogeneity demonstrated might serve the efficiency of the countercurrent mechanism with regard to urinary concentration The possibility is discussed that an active regulation of glomerular filtration and blood flow of the juxtamedullary nephrons may participate in the regulation of urinary concentration

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STUDIES ON THE PREPARATION OF COMPLEMENT FIXING POLIO ANTIGENS

By

JOHN PAPAPANAGIOTOU[1]

Received 11 ii 1961

The complement fixation (CF) technique has been applied for the demonstration of polio antibodies by many investigators. Suspensions of infected tissues of the central nervous system from mice (1, 2) and cotton rats (3–6) have been employed as sources of antigen. *Svedmyr et al.* (7) used as antigens tissue culture (TC) fluids infected with polio viruses and concentrated by ultrafiltration. *Black & Melnick* (8), *Miller & Baumeister* (9, 10), *Le Bouvier et al.* (11), and *Schmidt & Lennette* (12) also used infected TC fluids as antigens in CF tests. The latter authors (13) studied the factors influencing the potency of polio CF antigens produced in TC systems. *Hare & Warren* (14) reported that concentrates of poliovirus from TC fluid provide reagents of considerably enhanced antigenic activity in the CF test.

The purpose of the present study was to attempt to prepare suitable CF antigens from monkey kidney tissue culture (MK TC) fluids infected with the three types of poliovirus potent enough to be used routinely without concentration. In addition experiments were made to concentrate CF antigens in order to obtain antigens which were sufficiently potent to detect small amounts of CF antibodies in serum.

MATERIALS AND METHODS

Preparation of Tissue Cultures

The preparation of trypsinized monkey kidney tissue cultures has been described in detail previously (15). For virus production Roux flasks were seeded with 10^7 MK cells. The outgrowth medium consisted of 150 ml of medium 199 with 2 per cent horse serum.

Poliovirus Strains

The Brunhilde (Type I), MEF 1 (Type II) and Saukett (Type III) strains were used for the preparations of CF antigens.

The author wishes to express his indebtedness to Dr. *Herdis von Magnus* for suggesting this investigation for her continued interest in the work, as well as for her critical examination of the manuscript.

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Virus Titrations

The titrations were performed in MK tissue culture roller tubes (15). The medium during cell outgrowth consisted of 0.5 per cent of lactalbumin hydrolysate in Hanks' balanced salt solution with 2 per cent horse serum. Before inoculation this fluid was replaced by 1.8 ml of medium 199 (16).

Serial ten fold dilutions of the virus material were prepared in saline. Each dilution was inoculated into 10 tubes with an inoculum of 0.2 ml per tube. The tubes were incubated at 36° C in a roller drum. Final readings of the cultures were made 7 days after the inoculation of the virus dilution. Titers were calculated according to Karber's method (17, 18) and recorded as log of $TCID_{50}$ per 0.2 ml of the inoculum.

Hyperimmune Sera

Monkey sera. Polio antisera were prepared by a method described previously (16). The sera were stored at —20° C, and the same lots of monkey sera were used throughout this study. Before use in the CF test the sera were diluted 1:8 in veronal saline and inactivated at 60° C for 30 minutes.

The same 3 batches of monkey antisera (type I, type II and type III) were used throughout the study. They were kindly supplied by Dr. Annelise Godtfredsen of this institute.

Guinea pig sera. The guinea pig polio antisera were derived from animals used for the routine potency testing of polio vaccine. Guinea pigs weighing approximately 250 g were given either 0.2 ml of polio vaccine intradermally on days 1, 8 and 15 and bled on day 22, or 1 ml subcutaneously on days 1 and 15 and bled on day 22. The sera were stored at —20° C. Before use the guinea pig sera were inactivated at 56° C for 30 minutes in a dilution of 1:2 or 1:4.

Technique of Complement Fixation Test

The CF tests were performed in glass tubes (Widal tubes) with a total volume of 0.5 ml per tube (0.1 ml each of antigen, serum, complement, red cells and haemolysin).

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highest dilution (initial) showing a fixation more than 2 + (haemolysis less than 50 per cent) with the specific antigen.

Titrations of CF antigens

The CF antigens were titrated against the homologous monkey antisera in a 'chessboard' type titration The antigen undiluted and diluted 1 2 1 4 and 1 8 (or higher for the concentrated antigens) was titrated against 2 fold dilutions of the homologous serum

Each antigen dilution was checked for anticomplementary activity with 2 1 5 1 0 75 and 0 5 units of complement Controls consisting of serum dilution without antigen complement controls and a veronal buffered saline control were included in the test

One unit of antigen was defined as the dilution of the antigen (in 0 1 ml) which gave more than 2+ fixation (haemolysis less than 50 per cent)

EXPERIMENTAL

Effect of nutrient media In the initial experiments it was found that when Medium 199 had been used for outgrowth as well as for maintenance of the cultures, the infectious tissue culture fluid had a titer of less than 1 2 when used as antigen in CF tests It was therefore attempted to prepare more potent antigens by using other nutrient media These experiments were performed as follows

The medium used routinely for outgrowth of the cells in Roux flasks was—as mentioned previously—Medium 199 with 2 per cent horse serum In the present experiments this was changed after 4–7 days to a booster outgrowth medium, and the cells were allowed to grow for 4 more days At this time the latter medium was changed to a "maintenance medium" and the flasks were seeded with virus After 48 hours of incubation the fluids were harvested and used as antigens in CF tests

As "booster outgrowth medium" (in the following referred to as "outgrowth medium") 3 different media (A, B, and C) were employed For composition see Table 1

TABLE 1
'Outgrowth Media

	A	B	C
Hanks solution	ca 88*	ca 99	ca 90
Lactalbumin hydrolysate	0 5	0 5	0 5
Calf serum	2	0	10
Bovine amniotic fluid	10	0	0
Sodium bicarbonate	0	0 11	0 11
Yeast extract	0	0 1	0 1
Glucose	0	0 40	0 40
	100	100	100

* All figures are given in per cent

Five different maintenance media were tried The composition of Nos 1, 2, 3 and 5 are listed in Table 2 No 4 consisted of undiluted bovine amniotic fluid

TABLE 2

"Maintenance Media"

	1	2	3	5
Medium 199	100*	99	ca 80	77 5
Calf serum	0	1	0	0
Tryptose phosphate broth	0	0	20	20
Glucose –	0	0	0 40	0 40
Monkey serum	0	0	0	2 5
	100	100	100	100

* All figures are given in per cent

A number of experiments with various combinations of these "outgrowth media" and maintenance media was carried out, using the harvested virus fluids as antigen The results have been combined in Table 3 It will be seen that amongst the combinations tried "outgrowth media" A and C with maintenance medium 4 gave the most satisfactory polio CF antigens *Schmidt et al* (13) have previously employed medium C for preparation of CF antigens from HeLa cell cultures Medium 4, bovine amniotic fluids, was originally recommended by *Enders* (20) for use as tissue culture medium

TABLE 3

CF Antigen Titer Obtained with Various Media Combinations

		Maintenance Medium				
		1	2	3	4	5
Out	A	2*	2	1	2 4	2 (a)§
growth	B	1 2	1	1	1 2	2 (a)
medium	C	2	–	–	8	2 (a)

* Antigen titer 1 2

§ (a) indicates anti complementary effect of the antigen

From the ... ed preferable to change from ... with 2 per cent horse serum) ... the cultures were about 4 days old

In Table 4 various data from experiments with all 3 types of polio CF antigens are recorded The infectious titers obtained with media A and C were found not to be significantly different However, virus fluids from cells grown with medium C were consistantly found to yield a slightly better antigen than the fluids prepared with medium A This was for instance noted, when antigen dilutions of 1 8 were examined (these data are not included in the table)

The CF ...

noted from Table 4 that heterologous fixation occurred with all the antigens In the present experiments the heterologous serum titer was unusually high for type III antigen, 1.64 However, the heterologous serum titer was always significantly lower than the titer obtained with the homologous antisera

TABLE 4

Infectious Titers and CF Antigen Titers Obtained by Cultivation of Polio Virus in Various Nutrient Media

Polio virus type	Nutrient media used		Virus titer log TCD50 0.02 ml	CF titer of antigen	Anti complementary activity	CF titer of monkey antisera*		
	Out growth	Maintenance				I	II	III
I	A§	Bovine amn fluid	8 4	1 4	none	2048	8	8
	C§		8 1	1 4	none	2048	16	8
II	A	–	7 8	1 4	none	8	256	<8
	C	–	8 4	1 4	none	8	256	<8
III	A	–	7 8	1 2	none	64	16	1024
	C	–	7 7	1 4	none	64	16	1024

* Titers are expressed as reciprocal of serum dilution
§ For the composition of media A and C see text

Purification of CF Antigens

Fluorocarbon treatment Fluorocarbon has been used for purification of viruses by a series of authors (21–26) In the present experiments all operations were carried out under a hood equipped with ultraviolet lamps and an exhaust system One part of fluorocarbon (Freon 113[1]) was added to 9 parts of aqueous virus suspension, both cooled at + 4° C The two components were then blended at top speed (14,500 r p m) in a Servall Omnimixer for 3 minutes The blending vessel was kept immersed in an ice bath during the homogenization and subsequently allowed to stand undisturbed for 5 minutes in the ice bath Thereafter the homogenate was centrifuged at 1000 1500 r p m /10 minutes in a refrigerated centrifuge to separate the aqueous from the organic phase The supernate (aqueous phase) was harvested from the centrifuge tube and stored at + 4° C or — 20° C to be used as crude CF antigens At these temperatures the antigens were found to be stable for at least 6 months

A series of virus titrations was first carried out to determine the effect of fluorocarbon treatment on the infectious titer of all 3 types of poliovirus Table 5 shows that one treatment with fluorocarbon (1 part of Freon 113 plus 9 parts of infectious TC fluid) does not influence the virus titer of the fluids

1 Freon 113 (Trichlorotrifluoroethane $C_2Cl_3F_3$) E I Du Pont de Nemours & co

TABLE 5

Effect of one Fluorocarbon Treatment on the Infectious Titer of Poliovirus TC Fluid

Exp No	Polio antigen*		Log infectious titer (TCD50/0.2 ml)	
	Type	No	before treatment	after treatment
1	I	(3/11)	7.4	7.5
2	–	(29/12)	7.2	7.3
3		(547)	6.7	6.8
4	–	(0-118 A)	7.6	7.8
5		(0-118-B)	7.5	7.6
6		(0-118 C)	7.8	7.7
7	II	(3/11)	7.6	7.4
8		(533)	6.7	6.8
9	III	(10/11)	7.7	7.2
10		(557)	7.3	7.4
		Average	7.35	7.35

The polio antigens derived from cultures with medium 199 as maintenance medium The antigens in Exp 5 and 6 contained however 1 and 2 per cent calf serum respectively

TABLE 6

Effect of one Fluorocarbon Treatment on Polio CF Antigen

Antigen	Medium	+ Freon treatment	Antigen dilution	CF antibody titer with polio hyperimmune sera		
				Type I	Type II	Type III
Type I (118 A)	199	Untreated	1/1	4096	32	8
			1/2	4096	8	<8
			1/4	64		
		Freon treated	1/1	2048	<8	<8
			1/2	256	<8	<8
			1/4	<64		
Type I (0-136)	Bovine amniotic fluid	Untreated	1/1	2048	ND	ND
			1/2	1024		
			1/4	<64		
		Freon treated	1/1	1024	ND	ND
			1/2	256		
			1/4			

As mentioned previously polio CF antigens prepared in different media were not often anti C', and CF antigens prepared in bovine amniotic fluid were found to be practically free of any anti C' activity If, however the fluids were anticomplementary this effect could readily be removed by fluorocarbon treatment Also, the fixation with heterologous antisera which has been described in a previous section, was found to be removed or reduced by Freon treatment (Table 6)

While the fluorocarbon treatment of the antigens thus had a beneficial effect as regards cross reaction in the CF test, it was in the pre-

noted from Table 4 that heterologous fixation occurred with all the antigens In the present experiments the heterologous serum titer was unusually high for type III antigen, 1:64 However, the heterologous serum titer was always significantly lower than the titer obtained with the homologous antisera

TABLE 4
Infectious Titers and CF Antigen Titers Obtained by Cultivation of Polio Virus in Various Nutrient Media

Polio virus type	Nutrient media used: Outgrowth	Nutrient media used: Maintenance	Virus titer log TCD 50 0 2 ml	CF titer of antigen	Anticomplementary activity	CF titer of monkey antisera*: I	CF titer of monkey antisera*: II	CF titer of monkey antisera*: III
I	A§	Bovine amn fluid	8 4	1 4	none	2048	8	8
	C§	–	8 1	1 4	none	2048	16	8
II	A	–	7 8	1 4	none	8	256	<8
	C	–	8 4	1 4	none	8	256	<8
III	A	–	7 8	1 2	none	64	16	1024
	C	–	7 7	1 4	none	64	16	1024

* Titers are expressed as reciprocal of serum dilution
§ For the composition of media A and C see text

Purification of CF Antigens

Fluorocarbon treatment Fluorocarbon has been used for purification of viruses by a series of authors (21–26) In the present experiments all operations were carried out under a hood equipped with ultraviolet lamps and an exhaust system One part of fluorocarbon (Freon 113[1]) was added to 9 parts of aqueous virus suspension, both cooled at + 4° C The two components were then blended at top speed (14,500 r p m) in a Servall Omnimixer for 3 minutes The blending vessel was kept immersed in an ice bath during the homogenization and subsequently allowed to stand undisturbed for 5 minutes in the ice bath Thereafter the homogenate was centrifuged at 1000 1500 r p m./10 minutes in a refrigerated centrifuge to separate the aqueous from the organic phase The supernate (aqueous phase) was harvested from the centrifuge tube and stored at + 4° C or 20° C to be used as crude CF antigens At these temperatures the antigens were found to be stable for at least 6 months

A series of virus titrations was first carried out to determine the effect of fluorocarbon treatment on the infectious titer of all 3 types of poliovirus Table 5 shows that one treatment with fluorocarbon (1 part of Freon 113 plus 9 parts of infectious TC fluid) does not influence the virus titer of the fluids

1 Freon 113 (Trichlorotrifluoroethane $C_2Cl_3F_3$) E I Du Pont de Nemours & co

TABLE 8

Concentration of Polio CF Antigen by 4 Hours of Ultracentrifugation Effect of Gelatin

Exp No	Virus Type	Gelatin 0 06 %	Virus titer log TCD 50 0.2 ml				Factor of concentration
			Original	Supernatant		Sediment* resuspended	
				Upper 2 ml	Lower 10 ml		
A	I	+	6 8	4 2	-	8 1	20
		—	6 7	4 1	-	8 2	30
B	I	+		3 8	5 7	8 2	
		—		3 9	≧7 1	8 3	
C	II	+	6 8	5 1	5 5	8 4	40
		—		6 4	6 7	6 9	0
D	III	+	7 2	4 4		8 6	25

* *Sediment from each tube containing 30 ml was resuspended in 1 ml of Hanks' solution*

In introductory experiments the time factor was first examined by centrifugation for 2, 4 and 6 hours It was found that 4 hours of centrifugation was required for satisfactory sedimentation of the virus Next it was established that a gelatin (Difco) preparation employed in the concentration 0 06 per cent (27) was free of poliovirus inhibitors to type I and III, and had no untoward effects on the specific reaction of the tissue culture CF antigens (type II was not tested)

The gelatin (Difco) was prepared as a 6 per cent stock solution heated to 80° C for 20 minutes and filtered through an S-1 Seitz-type filter After centrifugation the supernatant was removed and the gel-like pellet was resuspended in warm (37° C) Hanks' solution to give the desired concentration, usually 30 or 10 times The faintly opalescent concentrated were then clarified by centrifugation at 2000 r p m./5 min It was found that placing the tube in a 37° C water bath for 5 minutes before centrifugation helped to dissolve the aggregates of gelatin

The result of the experiments with concentration of CF antigens in the ultracentrifuge has been shown in Table 8 At the end of each experiment the upper two and the last ten ml of the supernatant fluid as well as the sediment were collected The latter was resuspended in 1 ml The samples from each experiment were titrated in parallel on the same day and in the same batch of MK TC

It will be seen from Table 8 that a satisfactory concentration was obtained for type I, whether gelatin was added or not In the experiment with type II, however, a satisfactory concentration was obtained only in the tubes with gelatin added No data on centrifugation without gelatin are available on type III in the present series of experiments

The centrifugation technique described here proved to be very satisfactory for the concentration of viral antigens to be used in CF tests, and antigen titers of 1 32 were readily obtained when the fluids were

sent experiments observed that a reduction of the specific polio antigens also might occur during treatment with fluorocarbon

In Table 6 is shown this effect on 2 type I virus antigens, one grown in Medium 199 the other in bovine amniotic fluid

The addition of 1–2 per cent calf serum to Medium 199 had a certain protective effect on the CF antigen during the treatment with Freon 113 (24, 25) Examples of the results obtained in the present experiments are recorded in Table 7

TABLE 7

Protective Effect of Calf Serum on two Different Batches of Polio I Antigens during Treatment with Fluorocarbon

Medium	± Freon treatment	Antigen dilution	CF antibody titer with polio hyperimmune sera		
			Type I	Type II	Type III
Medium 199 (118 A)	Untreated	1/1	4096	32	8
		1/2	4096	8	< 8
		1/4	64		
	Freon treated	1/1	2048	< 8	< 8
		1/2	256	< 8	< 8
		1/4	<64		
Medium 199 with 1 % calf serum (118 B)	Untreated	1/1	2048	16	<16
		1/2	1024	<16	
		1/4	256		
	Freon treated	1/1	2048	<16	<16
		1/2	1024		
		1/4	<128		
Medium 199 (086 A1)	Untreated	1/1	2048	N D	N D
		1/2	2048		
		1/4	1024		
	Freon treated	1/1	2048	N D	N D
		1/2	1024		
		1/4	64		
Medium 199 with 1 % calf serum (086 A2)	*Untreated*	1/1	4096	N D	N D
		1/2	2048		
		1/4	1024		
	Freon treated	1/1	2048	N D	N D
		1/2	2048		
		1/4	512		

Concentration of polio CF antigen in the ultracentrifuge The purpose of the following experiments was to evaluate the suitability of CF antigens concentrated by ultracentrifugation The technique with the addition of gelatin before centrifugation has been described by *Baron* (27) and used by *Hare & Warren* for the concentration of poliovirus and of formalin treated polio vaccine (14)

The CF antigens were in the present experiments concentrated in a "Spinco" (Model L) ultracentrifuge in amounts of 30 ml per tube at 78000 × G (30,000 r p m)

TABLE 10

CF Titers of Type II Guinea Pig Polio Antisera Tested with Different Units of Type II CF Antigen

Antigen Type II	Units of CF antigen	CF titers of guinea pig type II sera*							
		4791	4792	4793	4795	4796	4798	4799	354
Crude	2	128	128	64	64	32	2	4	2
30× concentrate diluted 1 4	8	128	128	64	64	32	2	8	4

Titers as reciprocals of serum dilution

Finally experiments were carried out with a number of type III guinea pig sera In one experiment (A) 2 and 8 units of antigen were used while in another experiment (B) 2 and 4 units were employed Table 11 shows the results on 5 guinea pig sera and one serum pool which all were tested in both experiments

TABLE 11

CF Titers of Type III Guinea Pig Polio Antisera Tested with 2 4 and 8 Units of Antigen

Exp	Antigen	CF Units	CF titers on type III sera					
			9961	9962	9963	9965	9968	Pool [illegible]
A	0 1 ml crude	2	2	4	4	16	2	4
	0 1 ml (30 × conc) dilut	8	16	32	32	32	16	16
B	0 1 ml crude	2	2	4	4	8	2	4
	0 2 ml crude	4	8	16	16	32	8	16
	0 1 ml (30 × conc) dilut	8	N D	N D	N D	N D	N D	16

Legends see Table 9

By comparison of the results obtained with 2 units of antigen in experiments A and in B it will be seen that the serum titers were very reproducible in fact they were identical in the 2 experiments except for serum 9965 which showed a 2 fold difference in titer Furthermore experiments A and B showed that 4 units of antigen gave higher serum titers than 2 units and that the titers were still higher when 8 units of antigen were employed in the test

A series of type III antigens has been examined with ...

F ... the examination of t ... tests ... units of CF antigen are adequate for the detection of small amounts of CF antibodies In contrast for testing of type III sera it is desirable to employ 4–8 units of CF antigen

concentrated 30 times. (The supernatant fluids from the centrifugation were always found to be devoid of CF antigen.)

The concentrated CF antigens did, however, also react with the heterologous antisera, although a lower titer was obtained than with the homologous antigens.

The Effect of Varying Amounts of Antigen in CF Tests

During the experimental work some data had been obtained which indicated that while 1–2 units of polio CF antigen were sometimes satisfactory for CF titration also of guinea pig sera, better results might be obtained when more units of CF antigen were used in the tests. This problem was therefore examined for all 3 types of poliovirus.

In table 9 are shown the results obtained with CF tests on 7 type I guinea pig sera. In this experiment a crude type I virus suspension with a CF titer of 1:4 (*e.g.* 4 units per 0.1 ml) was used. These sera were tested against 2, 4 and 8 units of antigen, eight units of antigen being obtained by employing 0.2 ml of CF antigen instead of the usual volume of 0.1 ml. (This simple method for increasing the amount of polio CF antigens has been suggested by *Schmidt & Lennette* (12) who found that an increase in the total volume of the CF test from 0.5 ml to 0.6 ml had no significant effect on the CF antibody titers.) In the present experiment (Table 9) *it was found that the CF antibody titer of the type I guinea pig sera did not vary with the amount of antigen.*

TABLE 9
CF Titers of Type I Guinea Pig Polio Antisera Tested with Different Units of CF Antigens

Antigen type I crude	Units of CF antigens	CF titers of guinea pig sera Type I*						
		4811	4812	483	4837	4838	4839	4840
0.1 ml of 1:2 dilution	2	ND	16	<2	4	2	2	2
0.1 ml of undiluted	4	64	32	<2	ND	2	4	2
0.2 ml of undiluted	8	64	32	<2	4	4	4	2

* Titers as reciprocals of serum dilution.
ND = not done.

Subsequently eight type II guinea pig sera were tested against a crude type II Freon treated antigen with a titer of 1:2 (*e.g.* 2 units per 0.1 ml) as well as with a 30 times concentrate of a Freon treated antigen. This latter had a *titer of 1:32 and was used in a dilution of 1:4, e.g.* 8 units per 0.1 ml (Table 10). No difference in the titer of the guinea pig sera was seen whether 2 or 8 units of antigen were employed.

Another series of 10 type II guinea pig sera was tested against 2 and 4 units of CF antigen and again the same CF titer was *obtained independent* of the number of units of antigen employed.

TABLE 10

CF Titers of Type II Guinea Pig Polio Antisera Tested with Different Units of Type II CF Antigen

Antigen Type I	Units of CF antigen	CF titers of guinea pig type II sera*							
		4771	4712	4773	4775	4796	4798	4799	3?1
Crude	2	128	128	64	64	32	2	4	2
30 × concentrate diluted 1 4	8	128	128	64	64	32	2	8	4

* Titers as reciprocals of serum dilution

Finally, experiments were carried out with a number of type III guinea pig sera In one experiment (A) 2 and 8 units of antigen were used, while in another experiment (B) 2 and 4 units were employed Table 11 shows the results on 5 guinea pig sera and one serum pool which all were tested in both experiments

TABLE 11

CF Titers of Type III Guinea Pig Polio Antisera Tested with 2 4 and 8 Units of Antigen

Exp	Antigen	CF Units	CF titers on type III sera					
			9961	9962	9963	9965	9969	Pool 9921 30
A	0 1 ml crude	2	2	4	4	16	2	4
	0 1 ml (30 × conc) dilut	8	16	32	32	32	16	16
B	0 1 ml crude	2	2	4	4	8	2	4
	0 2 ml crude	4	8	16	16	32	8	16
	0 1 ml (30 × conc) dilut	8	N D	N D	N D	N D	N D	16

Legends see Table 9

By comparison of the results obtained with 2 units of antigen in experiments A and in B it will be seen that the serum titers were very reproducible, in fact they were identical in the 2 experiments except for serum 9965 which showed a 2-fold difference in titer Furthermore, experiments A and B showed that 4 units of antigen gave higher serum titers than 2 units and that the titers were still higher when 8 units of antigen were employed in the test

A series of type III antigens has been examined with various amounts of antigen in the CF test The above mentioned observations were found to apply to all type III antigens studied so far when these were used in CF tests against low-titered guinea pig antisera

From these experiments it therefore seems that for the examination of type I and II guinea pig sera in CF tests, 2 units of CF antigen are adequate for the detection of small amounts of CF antibodies In contrast, for testing of type III sera it is desirable to employ 4–8 units of CF antigen

DISCUSSION

Schmidt et al (13) reported the preparation of polio CF antigens from TC fluids, using HeLa, KB or embryonic intestinal cells in continuous culture These antigens could be used in CF tests without concentration In contrast, the above authors found that CF antigens from monkey kidney TC fluids were of rather low potency In our laboratory engaged in the preparation of polio vaccine, the monkey kidney TC cells were more easily available than HeLa cells or other cell lines and in the present study such cultures were therefore employed for the preparation of viral antigens In CF tests such antigens were in our laboratory found to have titers of 1 2–1 8, when suitable nutrient media were used

A serious problem in the preparation of CF antigens from TC fluids is the anti-C' activity of some of these antigens In the present experiments it was found that the use of bovine amniotic fluid as maintenance medium for the cultures resulted in antigens with no or only slight anti-C' activity *Svedmyr et al* (7) reported that the anti C' activity could be abolished by heating the preparation at 56° C for 30 minutes However, it was shown by *Le Bouvier* (28) and confirmed by *Schmidt & Lennette* (29) that the homotypic specificity of polio CF antigens is undesirably modified by heat, *i e* the antigens acquire during the heating a high degree of heterotypic activity

Hummeler &Hamparian (23) introduced treatment of the CF antigens with fluorocarbon for the removal of anti C' activity These and other authors (25) found that the medium employed for the preparation of the viral antigen was important for the preservation of the specific antigen during the Freon treatment In the present experiments it was found that antigens prepared in media containing 1–2 per cent calf serum was little damaged by Freon treatment

The examination of monkey hyperimmune sera in CF tests does not require the use of potent polio CF antigens However, when dealing with sera of low CF titer, as for instance sera from guinea pigs inoculated with formalin treated polio vaccine, it was found necessary to employ CF antigens of higher potency, and studies on the concentration of antigen in the ultracentrifuge were therefore carried out *Hare & Warren* (14) employed CF antigens concentrated in the ultracentrifuge for the study of polio antisera The concentration of poliovirus in the centrifuge is facilitated by the addition of gelatin to the TC fluid prior to the centrifugation as *Baron* has reported (27) This finding was confirmed in our studies During the centrifugation without gelatin a proportion of the type II virus was found not to be collected with the sediment but remained in the lower part of the supernatant (Table 8) Moreover, the sediment is more easily disrupted during the manipulations for the collection when gelatin is not used In the presence of gelatin a firm pellet is formed, visible in the bottom of the centrifuge tube

Employing different units of antigen in parallel titrations of the same immune guinea pig sera it was found that for the demonstration of type I and II polio CF antibodies the use of 4 or 8 units of CF antigen in the test was no better than 2 units of antigen (Table 9, 10). However, for the detection of type III CF antibodies 4 or 8 units did regularly (with all the type III antigens studied) give higher serum titers than did 2 units (Table 11). With the present knowledge on the properties of the three antigenic types of poliovirus it is not possible to explain this observation.

From our experience which conform with others (14) it seems that the type II poliovirus provides a good CF antigen and also that CF antibodies to type II are easily demonstrated. Poliovirus type I represents a less potent antigen and poliovirus type III is inferior in this respect to the other two types.

The present experiments confirmed the observation by others (12) that a double volume of infected TC fluids can be employed if a doubling of the antigen dose is required. Anti C' effect through the use of 0.2 ml instead of the usual 0.1 ml of Freon treated antigens was not encountered.

SUMMARY

Experiments on the production of CF polio antigens from monkey kidney tissue culture fluid virus using various culture media have been described. Results obtained with Freon treatment of the antigen for removal of anticomplementary activity and removal of what is presumably host antigen have been described. Also results of concentration in the Spinco ultracentrifuge with and without the addition of gelatin are recorded.

By the use of concentrated antigens it was demonstrated that as regards CF tests with type I and type II poliovirus, 1–2 units of antigen are sufficient. However, with type III it was found better to use 4–8 units of CF antigen, in that higher serum titers were obtained in tests with low-titered guinea pig sera, when the higher amount of antigen was employed.

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A NEW SALMONELLA SPECIES: SALMONELLA STRAENGNAES = 11:z_{10}:1,5

By

K ALIN, B BACKELIN, E BENGTSSON and I MATTSSON

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In November 1960 a Swedish UN force of about 600 men returned to its headquarters at Straengnaes The force had been serving abroad for about 6 months, the first 2 in the Gaza area of Israel and immediately after for 4 months in the Congo, mostly in the Katanga area

During the brief service numerous acute but slight cases of enteritis had occurred, but no one had been seriously ill On the return to Sweden none was suffering from enteritic symptoms As salmonella carriers were suspected a faecal specimen from every member of the force was cultured Twenty cultures yielded growth of different salmonella strains such as S amager, S chicago, S kisangani, S mampeza, S stanleyville, S typhi murium, S saint paul and S infantis

Four of the 20 strains proved to belong to a hitherto unknown species Two of the men from whom these strains had been isolated had shown signs of enteritis while in Gaza, the other two while in the Congo – only one of them with fever

Sera from 3 of the 4 men were tested for the presence of agglutinins towards the new type, all with negative results

Biochemically, the strains showed the following characteristics: rapid fermentation of arabinose, dulcitol, glucose (with gas), maltose, mannitol (with gas), rhamnose, sorbitol and xylose, no fermentation of lactose, salicin, sucrose and inositol, no production of indol, no decomposition of urea and no liquefaction of gelatine, positive reaction in Stern's glycerol fuchsin broth and in d tartrate, formation of H_2S, negative Voges-Proskauer reaction and positive methyl [illegible]

[illegible]

[illegible]

[illegible] straengnaes has been accepted

We are greatly indebted to Professor *F Kauffmann* for valuable help and confirmation of the antigenic formula

SUMMARY

The authors describe a new Salmonella species S *straengnaes* with the antigenic formula 11 z_{10} 1 5 Strains of this species were isolated from four U N soldiers on return from the Congo

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Kauffmann F Enterobacteriaceae Munksgaard I Copenhagen 1954

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PATHOPHYSIOLOGICAL INVESTIGATIONS INTO THE TERMINAL COURSE OF EXPERIMENTAL ANTHRAX IN THE RABBIT

By

B K NORDBERG, C G SCHMITERLÖW and H-J HANSEN

Received 4 ix 61

One of the characteristic features of *Bacillus anthracis* is its unusual reproductive capacity *in vivo* which explains how it can appear in enormous quantities in blood and other tissues This observation has long formed the basis of our conception of the mode of action characterizing this bacterium (*Toussaint* 1877) According to this conception *B anthracis* is believed to cause a capillary obstruction, in particular of the lungs This idea about asphyxia is also to be found in other interpretations of the pathophysiology of the disease So, *de Moulin* (1936) believed the asphyxia to be a sequel of some damage to the central nervous system In addition to these conceptions of the cause of death in *B anthracis* infection, more or less speculative though they are a few experimental pathophysiological papers have also been published in the literature of the early days *Resantzew*, for example, a Russian scientist in 1889 discovered that sheep dying of an experimental infection of anthrax showed anoxia in the terminal stage The O_2 concentration in the blood was only half the normal value In 1912, *Burow* published his results of haematological tests carried out during the various phases of experimental anthrax in rabbits guinea pigs, and mice He found a continuous reduction in the number of erythrocytes down to one third of the normal value when death occurred In addition he found poikilocytosis According to *Burow* the cause of death was obviously to be found in a hypoxaemia caused by haemolysis

Hence the early literature concentrated around two hypothetical modes of action of *B anthracis*, one of which was mechanical and the other chemical

According to *Topley & Wilson* (1955) the idea of bacteria exerting some sort of mechanical influence is no longer compatible with "our knowledge of the way in which the tissues deal with inert particles which have gained access to them This applies to bacteria in general As for anthrax *Smith et al* (1955 a) have shown that the fatal outcome

SUMMARY

The authors describe a new Salmonella species *S straengnaes* with the antigenic formula 11 z_{10} 1,5 Strains of this species were isolated from four U N soldiers on return from the Congo

REFERENCE

Kauffmann F Enterobacteriaceae Munksgaard F Copenhagen 1954

aged to prove the existence of a specific lethal factor in plasma from guinea pigs "dying of anthrax" Obviously, this factor is not identical with the substance from the capsule In 1958 the same team reported that they had been able to prove the existence of this toxin in *in vitro* cultures of noncapsulated bacteria

The morbid anatomy of both spontaneous and experimental anthrax is very little known, in the literature of the last years, however, there are two rather comprehensive studies of the pathologic anatomy of experimental anthrax First, *Ross* (1955) studied, from a histopathological angle, the guinea-pig material presented earlier that year by *Smith et al*, and a few years later (1959) *Cominsky* published a histological study of experimental anthrax in mice In both of these papers the interest is focussed on the presence of an extensive renal lesion as the most important change in a pathogenetic sense *Ross* considers the renal lesion in the guinea pigs to be of the same type as in "lower nephron nephrosis" but makes no indication as to the relative probability of the two main interpretations of its origin Either, she writes, "toxic action may be directly on the cells of the renal tubules, indirectly by some systemic action causing renal anoxia and so renal epithelial damage, or by a combination of these two factors" The "systemic action" of *Ross* is probably identical with the "secondary shock", the term used by her fellow-workers in other connexions Finally, she refers to the fact that *Berg et al* (1951) reached the same conclusion when studying the effect of Cl welchii toxin on the kidneys of dogs" *Cominsky* interprets her finding as follows the toxin probably destroys "the permeability of the inner glomerular membrane to pass to the tubules and bring about the necrosis there'

Obviously, there are still many points requiring elucidation in connexion with the damage caused to the organism by infection with *B anthracis* and finally leading to the animal's death Experimental anthrax in the rabbit being characterized clinically by asphyxia in its final stage, our work has concentrated on the terminal hypoxaemia and its causes The present paper is to give an account of that work

TECHNIQUE

A Throughout our investigations rabbits of 2 4 2 6 kg body weight have been used as experimental animals The animals have been infected by intravenous injection of a sporulating culture of *B anthracis* totalling 107–1 2 × 10

The infective agent h

B

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Fc

tre

sei

in the case of experimental animals is determined already at a stage when only 1/300 *of the terminal bacterial count* is reached Although streptomycin therapy after this critical stage may eradicate the bacteria, the animals do still die

The *modern interpretation of the mode of action of bacteria* rests on a chemical foundation: "The basis of all harmful effects of bacterial infection is quite certainly chemical" (*Topley & Wilson*) A considerable *number of attempts* have been made throughout the years to establish a production of endotoxin or exotoxin by *B anthracis* As for the earlier work in this field it is suggested that the reader consult the *comprehensive monographs published,* e g, *by Sobernheim* (1931) Quite recently much attention has been paid to the work carried out by two big teams, one American and one British, on the problem of the *production of toxins by the anthrax bacillus*

The American workers, *Watson et al* (1947), succeeded in isolating an "inflammatory factor" from tissue lesions caused by the bacillus and were *also able to prove that this substance possessed a* "tissue-damaging capacity in skin tests" *Nordberg* (1951) showed that filtrates from cultures of capsulated bacilli—as distinguished from filtrates of non *capsulated cultures—also had a "tissue-damaging capacity in skin* tests" However, *Watson et al* have not been able to establish any identity between their "inflammatory factor" and the substance (i e, poly-*d-glutamic acid) shown to be present in the capsule of B anthracis* (investigations by *Tomscik & Szongott,* 1933, *Ivanovics & Erdos,* 1937, *Ivanovics & Bruckner,* 1937) *King & Stein* in 1950 tried—though in vain—*to prove the existence of an active endotoxin and gloomily joined Bloom et al* (1947) in their statement "Our knowledge of anthrax in this respect (*i e*, its mode of action) has not been added to since the *turn of the century*"

When starting to attack the problem of "the chemical basis of the virulence of *B anthracis*" the British team around *Smith,* consequently, *had to set out from the fact that so far only factors of* a certain local tissue-damaging capacity had been shown to exist *Smith et al* started their work by preparing an extract of bacteria from *in vivo* cultures *and isolating* "extracellular products" from affected tissues However, they failed to prove the existence of any toxicity in these products (1953) Concluding that "the killing power of *B anthracis* is associated mainly with the growth of the organism in the tissues of the host" they then began to study the processes going on within the infected animal First, they carried out their work already mentioned on the terminal bacteraemia and then proceeded with clinical and morbid-anatomy studies of experimental anthrax in the rabbit These studies led to the conception of a secondary shock as the cause of death After this the investigations into the nature of the oedema-producing factor were resumed since that factor could be expected to "produce the vascular damage which leads to secondary shock *Smith et al* (1955) finally main-

aged to prove the existence of a specific lethal factor in plasma from guinea-pigs "dying of anthrax" Obviously, this factor is not identical with the substance from the capsule In 1958 the same team reported that they had been able to prove the existence of this toxin in *in vitro* cultures of noncapsulated bacteria

The morbid anatomy of both spontaneous and experimental anthrax is very little known, in the literature of the last years, however, there are two rather comprehensive studies of the pathologic anatomy of experimental anthrax First, *Ross* (1955) studied, from a histopathological angle, the guinea-pig material presented earlier that year by *Smith et al*, and a few years later (1959) *Cominsky* published a histological study of experimental anthrax in mice In both of these papers the interest is focussed on the presence of an extensive renal lesion as the most important change in a pathogenetic sense *Ross* considers the renal lesion in the guinea-pigs to be of the same type as in "lower nephron nephrosis" but makes no indication as to the relative probability of the two main interpretations of its origin Either, she writes, "toxic action may be directly on the cells of the renal tubules, indirectly by some systemic action causing renal anoxia and so renal epithelial damage, or by a combination of these two factors" The "systemic action" of *Ross* is probably identical with the "secondary shock", the term used by her fellow-workers in other connexions Finally, she refers to the fact that "*Berg et al* (1951) reached the same conclusion when studying the effect of Cl welchii toxin on the kidneys of dogs" *Cominsky* interprets her finding as follows: the toxin probably destroys "the permeability of the inner glomerular membrane to pass to the tubules and bring about the necrosis there"

Obviously, there are still many points requiring elucidation in connexion with the damage caused to the organism by infection with *B anthracis* and finally leading to the animal's death Experimental anthrax in the rabbit being characterized clinically by asphyxia in its final stage, our work has concentrated on the terminal hypoxaemia and its causes The present paper is to give an account of that work

TECHNIQUE

A Throughout our investigations rabbits of 2 4 2 6 kg

mainly consists of spores It has thus been possible to obtain a comparatively uniform infective dose for the investigations

The concentration of blood gases has been determined analytically according to the *van Slyke* method blood samples having been drawn from the carotid artery by means of a permanent cannula inserted under local anaesthesia Samples have been drawn both before the infection to check the normal values determined previously and at various stages of the infection In particular much interest has been devoted [illegible]

venous post mortem blood These samples have been taken from the right ventricle immediately after death The amount of blood gases in the arterial blood has also been studied in a similar manner in rabbits infected with other pathogenic micro organisms i e *Pasteurella multocida* and *Streptococcus pyogenes* and a crude toxin from *Clostridium novyi* the latter preparation being from a well known toxin producer (Cl novyi 2-A6) The titre of the crude toxin was 0 001 ml = LD_{100} in white mice of 14 16 gm body weight (intravenous injection) To further elucidate the matter the concentration of blood gases has also been determined in rabbits which have been mechanically strangulated under anaesthesia

The O_2 consumption by *B anthracis* has been studied in a series of investigations applying the *Warburg* technique The work has included determinations of the O_2 consumption by bacterial cultures in Ringer s solution and of the O_2 consumption in a mixture of aerated blood Ringer s solution and a culture of bacteria (1 part aerated blood + 4 parts Ringer s solution) To compare the results similar investigations have been made on other micro organisms both pathogenic and nonpathogenic

Determinations of the numbers of red and white corpuscles and the haematocrit value have been carried out according to standard techniques The standard values of each animal have been determined prior to infection and then by samples taken at regular intervals after infection and immediately after death For the sake of comparison the number of blood cells and the haematocrit value have been determined likewise in cases of infection caused by *Pasteurella multocida* and of injection of *Clostridium novyi* toxin Further these values have been determined when the infective agent has been added to normal blood *in vitro* after incubation for 6 hr at 37° C

The osmotic resistance of erythrocytes has been investigated through a number of experiments erythrocytes from both normal and infected rabbits having been analyzed Blood samples have been taken both before infection and in immediate connexion with the death of the animal

In another series of experiments the O_2 consumption in liver slices in the presence of a *B anthracis* culture has been studied by means of the *Warburg* technique The liver slices used have been prepared from livers of healthy rabbits

At various points during the course of their disease infected rabbits have been treated with a single dose of benzyl penicillin (100 000 international units per kg body weight intravenously)

B Studies of the morbid anatomy including a full post mortem gross examination and a histological examination of the spleen an abdominal lymph gland the trachea lung stomach intestine liver kidney myocardium skeletal muscle brain and adrenal gland have been carried out not only on 20 animals belonging to the series already mentioned but also on a series of 15 animals infected according to the same technique but never used for any purpose other than the morbid anatomy examination Each autopsy has been completed within 1 hr of the animal s death which generally occurred 25 55 hr after the infection

In the latter group each of 5 rabbits out of the 15 has been treated with 100 000 international units of penicillin per kg body weight some 1 or 3 hr before death in order to study the pathological picture of a terminal stage with no bacteria present Nevertheless the rabbits died just as easily

The following fixation fluids have been used A 10 per cent aqueous solution of formaldehyde a 20 per cent aqueous solution of formaldehyde during 15 minutes of cautious heating to 70 80° C— rapid fixation Helly s fluid Carnoy s fluid and Stieve s fluid

The following staining methods have been employed Haemalum eosin haematoxylin picric acid fuchsin (van Gieson s stain) PAS staining according to Hotchkiss Scarlet Red Alcian Blue methyl green pyronine (Unna Pappenheim) Ladewig s

mod f cation of Mallory s An l ne Blue collagen stain Hueck s mo lificati n of Tir mann Schmelzer s Turnt ull s Blue l epchne s l enzl line test an l 0 1 per cent aqueous soluti n of Tol udine Blue buffere l at vari us pH 0 1 per cent I nceau S l uffered at var ous pH

RESULTS

1 *Correlation between Clinical Manifestations and Bacteraemia*

It has been established that all animals in the series under test have actually died from anthrax within 2) 55 hr after being infected In no case has it been possible to establish a peracute or chronic course of the disease nor has any haemorrhage from the anus or the naso-pharyngeal cavity been observed From blood films it was found that the bacteria first appeared in the blood 20 32 hr after the moment of infection

Together with the bacteraemia the animals showed a rise in temper ature which in some cases was considerable and less marked in others The main clinical manifestation was an increasing dyspnoea in the latter part of the process

It was found in all animals that during the first stage of bacteraemia the blood contained only noncapsulated anthrax bacilli initially as soli tary bodies but later on when the temperature got higher in increasing numbers Immediately after the pyrexia had reached and passed its maximum the microbes began to appear in their capsulated form In a number of cases the capsules disappeared again for a limited period or their frequency was reduced for a short time generally in connexion with a new rise in temperature later on when the temperature dropped they would occur again in great numbers

Furthermore microscopical examination of smears of blood with drawn during the decline of pyrexia revealed a partial bursting of the capsule in some of the microbes This process generally occurred when the declining temperature approached or fell below the normal body temperature typical of that individual animal The bursting of capsules advanced slowly and at short intervals in the final stage immediately before death when there were numerous bacilli in the blood stream the bursting of capsules increased to such an extent that the surround ing medium *i e* the blood seemed to be penetrated with the contents of the capsules This could be seen from blood smears stained according to *Heim* with old methylene blue dye at the moment of death or imme diately afterwards as a reddish violet mass surrounding the microbes which on this occasion were noncapsulated The same colo [illegible]

[illegible] u um (i e , blood) surrounding the mi crobes assumed a light blue hue

The correlation between the body temperature of the sick animals and the developmental phase of the microbes in the blood was clearly noticeable During the rise in temperature noncapsulated microbes

alone are to be found whereas capsulated forms occur during the fall in temperature

The haemato-morphological examinations of blood from infected rabbits showed that samples taken 3 5 hr *prior to death contained capsulated B* anthracis microbes However, it was not possible to establish the presence of any free capsular substance and the erythrocytes showed quite a normal appearance

Samples taken 1 hr before the animal's death proved to contain numerous microbes with ruptured and partly defective capsules, ample quantities of capsular fragments of varying sizes, and liberated capsular substance

Blood samples drawn during the preagonal stage showed great numbers of microbes and of capsular fragments and substance The staining properties of the erythrocytes varied considerably Those that took the stain in the usual way also showed normal shape and size A great number of red blood cells remained almost unstained, in addition, and contrary to the corpuscles showing normal staining properties, they were characterized by a significant increase in diameter size The presence of abnormal erythrocytes as described is typical of blood in a haemolytic state There were also many corpuscles of normal size but of impaired staining properties (reduction of haemoglobin content) This blood showed the same picture despite 15 minutes' aeration during *which it assumed a light red colour By phase microscopy of preparations* in a native state it was noticed that part of the erythrocytes had assumed a spherical shape that clearly differed from the normal biconcave form

Examination of blood samples from rabbits that had been inoculated with Pasteurella multocida and from others that had been given toxin from Clostridium novyi at no stage of the disease revealed any changes in the appearance of the red blood cells, nor could any signs of haemolysis be detected

2 *Content of Blood Gases in the Course of Disease*

The normal average blood-gas concentration as determined in 24 rabbits was found to be 11 6 per cent (by vol) of O and 42 9 per cent (by vol) of CO_2 in arterial blood, and 8 2 per cent (by vol) and 38 9 per cent (by vol), respectively, in venous blood On the whole these blood gas values show great individual differences, probably due to the fact that there are considerable variations *in the pulmonary ventilation* in the rabbit, particularly as concerns the breathing frequency

The determination of blood gases according to *van Slyke* requiring *a comparatively* large quantity of blood for each test, it has not been possible to carry out a continuous blood gas analysis in each animal For this reason samples have been taken in a series of 10 rabbits, at various times before death (the time range being from 5 hr to 5 min) The results are presented in Table 1

TABLE 1

Concentration of O_2 and CO_2 in Arterial Rabbit Blood at various Stages of Infection

Rabbit No	Time of sampling (min before death)	Relative frequency of microbes in blood at sampling	O_2 in blood (per cent by volume)	CO_2 in blood (per cent by volume)
117	5	(+)(+)(+)	0.3	14.0
110	8	(+)(+)(+)	0.6	15.0
167	10	(+)(+)	1.7	36.7
171	18	(+)	4.7	48.2
172	30	(±)	9.0	44.8
109	40	(±)	10.1	29.6
2658	60	+	11.4	21.5
173	75	+	12.3	21.2
169	90	+	10.5	37.3
170	300	±	11.8	34.8

Explanations + non capsulated B. anthracis
(+) capsulated B. anthracis

TABLE 2

Concentration of O_2 and CO_2 in Blood from Rabbits Inoculated with B. anthracis

Rabbit No	Arterial Blood				Venous Blood	
	O_2 (% by vol.) p.i.	O_2 (% by vol.) p.a.s.	CO_2 (% by vol.) p.i.	CO_2 (% by vol.) p.a.s.	O_2 (% by vol.) p.m.	CO_2 (% by vol.) p.m.
112	13.6	0.3	40.5	27.6	0.2	29.0
114	14.7	0.4	38.6	27.6	0.1	29.7
115	13.9	0.3	36.0	15.0	0.1	29.0
116	12.1	0.1	38.1	15.1	0.1	18.2
118	12.0	0.1	37.6	12.1	0.1	16.7
119	11.3	0.2	44.3	20.8	0.1	27.2
120	10.1	0.2	34.0	13.7	0.1	18.7
121	11.7	0.1	35.1	23.7	0.1	30.3
123	13.6	0.1	38.0	20.2	0.1	24.8
124	10.7	0.1	37.3	18.6	0.1	21.8
125	12.7	0.1	45.8	24.2	0.1	26.6
Mean	12.4	0.2	38.7	19.9	0.1	24.7

Explanations p.i. = prae infectionem
p.a.s. = pre agonal stage
p.m. = post mortem

It can be seen from the Table that both O_2 and CO_2 values are particularly low just before death. The reduction of O_2 is noticeable already some 30 min. before death, reaching extreme values 10–5 min. before.

In the following experiments we have thus concentrated on determining the O_2 and CO_2 values immediately before death. The results are to be found in Table 2.

From Table 2 it can easily be seen that there is a very marked hypoxaemia in the preagonal stage of anthrax infections. The drop in CO_2 content, although significant, does not produce the same low values as does the O_2 content. On the whole the O_2 content of the venous blood

alone are to be found whereas capsulated forms occur during the fall in temperature

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The determination of blood gases according to *van Slyke* requiring a comparatively large quantity of blood for each test, it has not been possible to carry out a continuous blood-gas analysis in each animal For this reason samples have been taken, in a series of 10 rabbits, at various times before death (the time range being from 5 hr to 5 min) The results are presented in Table 1

The experiments with Streptococcus pyogenes have yielded results quite analogous to those described for Pasteurella multocida inoculations. So high O_2 and CO_2 values have been obtained and there is a great difference between the blood O_2 of anthrax and streptococcus inoculated rabbits. The results are summarized in Table 4.

In the pre agonal stage the O_2 concentration in the blood of rabbits inoculated with Clostridium novyi toxin was less than in animals inoculated with the two last mentioned bacteria, yet much higher than the values obtained from blood after inoculation with B. anthracis. The results, as may be seen from Table 5, indicate that in the pre agonal stage the CO_2 concentration, under the influence of a toxin of this kind, approaches the values that are normally found in non infected rabbits.

To further increase our comparative material we have strangled anaesthetised rabbits. The results can be seen in Table 6.

TABLE 5

Concentration of O_2 and CO_2 in Blood from Rabbits Following i.v. Injection of Clostridium novyi Toxin

Rabbit No	Arterial blood				Venous blood	
	O_2 (% by vol.)		CO_2 (% by vol.)		O_2 (% by vol.)	CO_2 (% by vol.)
	p.i.	p.a.s.	p.i.	p.a.s.	p.m.	p.m.
146	13.8	2.7	32.2	36.6	0.4	41.7
148	12.5	7.0	42.8	37.9	0.7	38.9
150	11.8	4.8	37.3	26.5	0.3	30.9
151	11.4	5.6	31.8	37.7	0.4	40.8
155	13.5	7.1	35.4	27.8	0.6	29.2
161	13.6	8.3	34.9	28.5	0.5	30.8
162	11.9	3.0	32.1	32.7	1.0	33.3
163	13.2	6.1	39.8	34.5	0.4	39.4
164	13.0	5.0	38.0	40.4	0.4	40.5
165	8.8	3.9	37.7	24.9	0.5	33.5
166	12.2	8.4	37.0	33.8	0.4	35.6
Mean	12.3	5.6	36.2	32.8	0.5	35.8

Explanations: Cf. Table 2.

TABLE 6

Concentration of O_2 and CO_2 in Arterial Blood from Non Infected Rabbits Strangled under Anaesthesia

Rabbit No	O_2 (% by vol.)		CO_2 (% by vol.)	
	normal	p.a.s.	normal	p.a.s.
205	13.2	8.4	27.3	38.4
206	11.7	2.8	33.4	34.4
207	10.6	6.2	25.6	40.1
208	10.2	8.9	42.4	43.4
209	10.9	4.0	46.4	41.6
210	14.8	6.3	46.4	39.1
Mean	11.9	6.1	36.9	39.5

Explanations: p.a.s. = pre agonal stage.

seems to follow the arterial value whereas the venous CO_2 content is generally higher than the corresponding arterial value.

For the sake of comparison we have examined the blood gas content in rabbits inoculated with *Pasteurella multocida*. The results of these investigations will be found in Table 3.

TABLE 3
Concentration of O_2 and CO_2 in Blood from Rabbits Inoculated with P. multocida

Rabbit No.	Arterial blood				Venous blood	
	O_2 (% by vol.)		CO_2 (% by vol.)		O_2 (% by vol.)	CO_2 (% by vol.)
	p.i.	p.a.s.	p.i.	p.a.s.	p.m.	p.m.
132	13.4	11.9	35.1	13.4	0.5	23.0
133	11.8	12.2	35.2	12.2	0.1	18.8
134	12.4	7.8	38.8	10.9	0.3	20.4
135	11.7	9.0	38.0	16.7	0.1	16.0
137	12.2	8.0	35.7	11.5	0.1	21.5
138	13.2	9.5	35.9	19.6	0.3	17.0
139	12.4	9.7	34.8	16.6	1.0	17.3
142	12.3	8.2	31.7	10.1	0.1	19.6
144	12.0	9.9	36.1	16.5	0.5	10.7
145	14.5	9.8	30.6	17.5	0.2	18.0
Mean	12.6	9.6	35.7	14.5	0.3	18.2

Explanations: Cf. Table 2.

In this series only a slight reduction of the O_2 concentration occurs in the pre-agonal stage. As can be seen from the Table, there is a considerable difference in the values of anthrax and pasteurella inoculated rabbits. There is a feeling that the drop in O_2 in the latter group may be referred to the bad general condition of the animals (impaired circulation, low vital capacity, etc.) whereas in the anthrax rabbits the seriously low O_2 concentration must have quite another, and basically different, background.

TABLE 4
Concentration of O_2 and CO_2 in Blood from Rabbits Inoculated with Streptococcus pyogenes

Rabbit No.	Arterial blood				Venous blood	
	O_2 (% by vol.)		CO_2 (% by vol.)		O_2 (% by vol.)	CO_2 (% by vol.)
	p.i.	p.a.s.	p.i.	p.a.s.	p.m.	p.m.
112/s	17.6	11.3	33.1	25.4	1.2	18.8
117/s	13.0	9.7	44.3	10.1	0.8	23.7
103/s	14.4	6.0	30.2	21.9	1.0	19.6
111/s	16.1	9.6	40.9	17.5	0.5	21.4
114/s	11.7	13.9	36.2	12.0	3.9	28.8
Mean	14.5	10.1	36.9	17.4	1.5	22.5

Explanations: Cf. Table 2.

Oxygen Consumption by B anthracis

Tests have been carried out according to the Warburg technique to find out whether the low O_2 concentration in blood taken from anthrax-infected rabbits in the pre-agonal stage may be caused by a big consumption of O by the microbe itself The consumption of O_2 was determined by mixing the culture of bacteria, after 12 hr incubation at 37° C and centrifugation with a liquid comprising aerated rabbit blood (from non infected animals) in Ringer s solution (1 in 4) These tests showed a high O consumption not only in the case of B anthracis but also when other bacteria were present in aerated blood A low consumption of O_2 was found only in the case of certain apathogens as compared with the pathogens under test

Determinations of the O_2 consumption by cultures of bacteria in the absence of blood indicate that the biggest O_2 consumption occurs with B anthracis A low O_2 consumption was found with Pasteurella multocida whereas intermediate values were found for the other pathogens under test

From these tests it might be justified to conclude that the very low O_2 concentration in the blood of anthrax infected rabbits cannot be caused by the O_2 uptake of the anthrax bacillus itself since, *e g*, Pasteurella multocida and Staphylococcus spp show about the same O_2 consumption in the presence of blood

Effect of B anthracis on the Hepatic O_2 Consumption

In order to determine whether B anthracis interferes with the metabolism of the individual organs a number of tests have been carried out in which the O_2 consumption of the liver has been studied by the Warburg technique The tests have been performed on slices of fresh rabbit liver the normal O consumption of which has been determined in Ringer s solution Liver slices have then been incubated with cultures of B anthracis It was not possible, however, to find any definite effect of the microbes on the O_2 consumption of the liver slices

Variations in Blood Cell Count and Haematocrit Value

Already during our first experiments with anthrax infection in the rabbit it was found that, in the final stage of the disease, their blood showed obvious signs of haemolysis A further study of this phenomenon has been carried out by haematological counts and haematocrit [illegible] the

[illegible] there is a decrease in the number of red cells when the rise in temperature has reached its maximum, the body temperature has begun to fall and the microt [illegible]

As can be seen from Table 6, O_2 values as low as those found in animals dying from anthrax do not occur even after mechanical strangulation

Furthermore, the blood-gas content of standard blood has been investigated following *in vitro* inoculation (syringe with mercury seal) with B anthracis cultures The results of these investigations are summarized in Table 7 From the Table it is evident that in infected blood samples stored for 6 hr at 37° C there is a considerable reduction in the concentration of O_2 as contrasted with non-infected samples Further, it is evident that inoculated blood samples stored at 4° C during the same period of time show no significant changes in the blood gas concentration as compared with non-infected samples Hence it seems justified to say that the concentration of blood gases does not vary during 6 hr storage at + 4° C

TABLE 7
Concentration of O_2 and CO_2 in Blood Following in vitro Inoculation with B anthracis

Sample No	Infected		Non Infected		Samples taken after
	O_2 (% by vol)	CO_2 (% by vol)	O_2 (% by vol)	CO_2 (% by vol)	
1	2 0	55 7	10 4	46 7	6 hr at 37° C
2	2 7	53 8	15 8	42 8	
3	2 5	36 7	11 5	27 7	–
1	8 2	39 8	8 1	30 6	6 hr at 4° C
2	10 2	43 1	10 1	39 6	–
3	9 7	44 3	9 2	43 2	–

The exceptionally low pre-agonal concentration of O_2 in the blood that had been inoculated with anthrax led to another investigation to establish whether the dark blood in question had lost its O_2 binding capacity completely To this end the infected blood was exposed to atmospheric oxygen by rotating it in an Erlenmeyer flask

We then found that after 7 15 min the blood assumed a light red colour When measuring the O_2 content of this blood according to van Slyke the O_2 values were again similar to those found in blood from non-infected rabbits (*i e*, *normal values*) *It appeared that the blood* was capable of maintaining these normal values for 30 min after which there was a gradual drop in O_2 and after 100 min the abovementioned exceptionally low O_2 values were reached

The CO_2 concentration of the infected blood decreased as the O_2 increased and then started to rise as the O_2 value sank It should not be forgotten that the respiration of the microbes in the blood may play an important rôle in the above mentioned processes

tocrit value The leucocyte number, on the other hand, after a temporary drop seems to increase in the pre-agonal stage

A comparative investigation of the corresponding values in rabbits inoculated with Pasteurella multocida or injected with Clostridium novyi toxin has given the results summarized in Table 9 As can be seen from this Table there is no real drop in the number of blood corpuscles nor in the haematocrit values

TABLE 9

Number of Erythrocytes and Leucocytes and Haematocrit Value of Rabbit Blood Following Inoculation of Pasteurella multocida or Injection of Clostridium novyi Toxin

Rabbit No	Sample taken	Body temp	Erythrocytes mill cu mm	Leucocytes cu mm	Haematocrit value	Agent
8 59	p i	39 0	6 0	6400	26	Past multocida
		40 6	6 0	3400	26	
	p a s	41 4	6 0	4000	33	
81/59	p i	39 2	6 6	9700	36	Past multocida
		40 3	6 6	10000	36	
		41 4	6 5	8300	36	
	p a s	40 6	6 0	5900	39	
12/59	p i	39 8	4 7	6400	21	Past multocida
		40 0	4 5	4200	22	
	p a s	39 9	4 9	3000	24	
13 59	p i	39 1	5 5	6000	30	Past multocida
		41.2	5 3	8300	30	
	p a s	40 5	5 8	10800	32	
14 59	p i	39 0	7 6	8400	36	Past multocida
		40 1	7 3	9000	36	
	p a s	39 6	7 6	9100	37	
19	p i	39 3	7 1	7600	33	Clostr novyi toxin
	p a s	39 0	7 0	7800	34	
20	p i	39 0	7 2	6900	36	Clostr novyi toxin
	p a s	39 0	7 3	6400	39	

Explanations p i = prae infectionem
p a s = pre agonal stage
No hemolysis

From Table 10 it can be seen, however, that in the *in vitro* experiments (syringe with mercury seal) with B anthracis infection summarized there, conditions are similar to *in vivo* conditions

As can be seen from Table 11 the values of the osmotic resistance of the erythrocytes in normal rabbit blood range between 0 20 and 0 50 per cent in infected rabbits the corresponding values range between 0 20 and 0 75 per cent This shows that the osmotic resistance of the erythrocytes has undergone a significant reduction, and this fact explains the low values of the erythrocyte numbers in the experiments

TABLE 8

Number of Erythrocytes and Leucocytes and Haematocrit Value of Rabbit Blood Following Inoculation of B anthracis

Rabbit No	Sample taken	Body temp	Erythrocytes mill cu mm	Leucocytes /cu mm	Haematocrit value	Smear test
11	p i	39 1	7 0	9600	40	—
		40 0	7 1	15000	34	+
		39 2	3 8	4600	28	(+)
	p a s	37 3	3 6	13900	8	(+)(+)
12	p i	39 6	6 1	10900	42	—
		40 1	6 7	17000	30	+
		39 3	4 9	13300	30	(+)
	p a s	36 0	2 5	17300	10	(+)(+)
6	p i	38 9	5 1	8700	29	—
		39 8	5 1	13000	29	+
		38 2	5 0	7200	25	(+)
	p a s	37 1	2 6	16500	16	(+)(+)
9	p i	38 8	5 0	6800	32	—
		40 0	5 6	16900	27	+
		37 5	4 5	10500	27	(+)
	p a s	36 8	3 5	29800	20	(+)(+)
177	p i	39 0	5 4	7600	28	—
		40 2	5 6	16500	28	+
		39 5	5 0	6500	28	(+)
	p a s	38 2	3 6	16700	16	(+)(+)
175	p i	39 2	5 3	8300	33	—
		40 2	5 5	18000	23	+
		37 8	3 4	12500	20	(+)
	p a s	36 8	3 0	18500	13	(+)(+)
174	p i	38 6	5 3	5800	34	—
		40 1	5 5	15300	28	+
		39 2	3 4	10800	20	(+)
	p a s	36 8	2 0	21200	12	(+)(+)
16	p i	39 3	6 1	5200	34	—
		41 0	6 4	18300	32	+
		38 6	5 4	7900	28	(+)
	p a s	36 8	3 8	15400	21	(+)(+)
13	p i	39 1	5 5	11200	37	—
		41 2	6 1	17300	33	+
		38 2	5 6	15800	28	(+)
	p a s	37 1	2 3	16400	10	(+)(+)
203	p i	39 2	6 5	5500	43	—
		39 8	6 7	16800	38	+
		38 1	4 6	10200	30	(+)
	p a s	37 0	2 8	18600	15	(+)(+)

Explanations p i = prae infectionem
p a s = pre agonal stage
— = no B anthracis found
\+ = B anthracis noncapsulated
(+) = B anthracis encapsulated

quantities in the blood of anthrax cases would cause a capillary obstruction, particularly pulmonar, leading to the asphyxia well known in anthrax

TABLE 11

Conditions of Osmotic Resistance of Rabbit Erythrocytes in B anthracis infection

Rabbit No	Sample taken	Osmotic resistance
11	p i	0 20 – 0 48
	p a s	0 20 – 0 78
12	p i	0 20 – 0 56
	p a s	0 20 – 0 76
6	p i	0 20 – 0 50
	p a s	0 20 – 0 78
9	p i	0 20 – 0 56
	p a s	0 20 – 0 72
177	p i	0 20 – 0 52
	p a s	0 20 – 0 70
175	p i	0 20 – 0 48
	p a s	0,20 – 0 70
174	p i	0 20 – 0 48
	p a s	0,20 – 0 76
16	p i	0 20 – 0 48
	p a s	0 20 – 0 78
13	p i	0 20 – 0 46
	p a s	0 20 – 0 78
203	p i	0 20 – 0 46
	p a s	0 20 – 0 70

Explanations p i — prae infectionem
p a s — pre agonal stage

Investigations into the Morbid Anatomy of Anthrax

Gross Pathology

There has been great uniformity in our findings as concerns the morbid anatomy of experimental anthrax in the *rabbit* Put together these structural findings form a characteristic pattern independent of whether the animals have died without any treatment or if the bacteria have been eradicated by penicillin therapy in the last stages of the disease For this reason we need not make any difference between, or within the two groups in this report

The *spleen* is generally slightly ...
dark red pulp The ...
are less clear, a slig...

A constant phen... ...ytracheitis, sometimes very strong, associated with oedema of the *trachea*, a condition reaching about 1 cm into the main bronchi where it may often come to a sudden stop In a few cases we have also found small submucous haemorrhages The

TABLE 10

Number of Erythrocytes and Haematocrit Value of Rabbit Blood Following in vitro Inoculation of B. anthracis

Blood sample No.	Erythrocytes mill./cu. mm		Haematocrit value	
	With anthrax	Without anthrax	With anthrax	Without anthrax
1	7.6	7.6	42	42
	6.3	7.6	33	35
	5.2	7.5	20	28
2	4.8	4.8	35	35
	5.0	5.3	20	25
	3.3	4.7	14	25
3	7.8	7.8	39	39
	6.7	7.6	33	39
	6.3	7.0	22	27
4	7.8	7.8	36	36
	6.7	8.0	34	34
	5.6	7.7	16	18
5	7.2	7.2	38	38
	5.9	7.2	33	34
	5.4	7.2	30	35
6	6.8	6.8	36	36
	6.1	6.9	36	38
	5.9	6.0	22	30

The samples were incubated for 6 hr. at 37° C.

Effect of Penicillin on the Course of Disease

A number of infected rabbits have been treated at various stages of the disease with a single dose of benzyl penicillin (100,000 I.U./kg body weight).

We then found that all rabbits treated with benzyl penicillin before, or soon after, the culmination of the temperature would recover whereas the rabbits treated at a later stage when the microbes in their blood had assumed the capsulated form did not survive.

Microscopic examination of material taken from the liver, spleen and intracardial blood revealed no microbes in a vegetative state. Smears, on the other hand, showed plenty of capsular substance from microbes whose cellular structure had been destroyed.

When cultivating material on beef broth agar, however, isolated colonies of B. anthracis were found in 25 per cent of the cases, and more precisely when material from the spleen or liver was being cultivated. There is the possibility of the microbes persisting in these organs as spores, however, none of the many smears made from the organs revealed the presence of any vegetative forms.

As already pointed out by *Smith et al.* (1955) there are strong reasons to revise the theory launched by early workers in this field (*Toussaint* and others) according to which the microbes present in ample



Figs 1–2

Fig 1 O 5397/59 Rabbit kidney Hematoxylin eosin 300 × Bacterial masses in the glomerular space with heavy compression of the tuft

Fig 2 P 238 60 Rabbit kidney Hematoxylin eosin 300 × Different types of tubular derangement Strongly acidophil casts



Fig 3

a) and b) O 5396/59 Rabbit kidney Ladewig 125 × c) P 238/60 Rabbit kidney Hematoxylin eosin 125 × Note the presence in the glomerular space and in the tubules of strongly acidophil casts and the different types of tubular degeneration

interalveolar and in the peribronchial and perivascular connective tissue or as more circumscribed infiltrations, particularly in the inter lobular interstice The vessels on the other hand show an obvious corresponding granulocytosis and it is often possible to follow the very migration of the granulocytes even through arterial walls of considerable thickness surrounded by particularly ample infiltrations In addition to the infiltrations of granulocytes the material generally also shows a rather distinct hyperplasia of the peribronchial lymph nodes Emphysema atelectasis and oedema are found in places, and in some minor sections there are signs of an acute serous pneumonia with great numbers of granulocytes in the exudate and also some desquamated epithelia The exudate, as well as the oedema in many cases, is strongly acidophil obviously a result of haemolysis

lungs have shown various degrees of hyperaemia, oedema and emphysema and, in addition, haemorrhages in a few cases. The oedema of the trachea and lungs, which remains moderate in the newly dead animal, becomes strongly increased if the body is left for 12–24 hr prior to the autopsy (a similar post-mortem development of an oedema of the lungs has been described by *Holmstedt et al.* in 1957 from experimental poisoning of mice with cholinesterase inhibitors). In a few cases we have found slight consolidations of the type known from pneumonia. In the pleurae and other *serous cavities* a moderate, thin transudation has been seen in a few cases, sometimes with an admixture of fibrin or blood. *Subepicardial* and *subendocardial* petechial haemorrhage is a condition rather often seen.

There is often a *retropharyngeal oedema*. The *gastric mucosa* generally shows the picture of an oedema with haemorrhages or haemorrhagic erosions of the fundus portion. In a few cases even the *intestinal wall* has been slightly oedematous.

No definite gross changes have been found in the parenchymatous organs: *liver, kidneys, and myocardium*. When urine has been present in the bladder the liquid has been dark, reddish, and sometimes definitely mixed with blood.

Microscopical Appearances

The *spleen* in each case presents the picture of an acute splenitis with a considerable enlargement of the reticulum of the red pulp and also with a rather widespread necrosis of the follicles. The abundant inflammatory oedema stores not only leucocytes but also large numbers of anthrax bacilli; in fact, the bacilli occur in such quantities that it seems justified to make them responsible for the greater part of the enlargement of the organ. They have generally been found in very large quantities in all organs subjected to microscopical examination, with the exception of the brain, in particular in the spleen and lymph-nodes but also in the renal glomeruli, lungs, and liver.

The *body lymph-nodes* show the same basic picture of a grave acute inflammation. In many cases extensive necrosis of the follicles has been observed. The most constant features, however, are hyperaemia and an oedema which generally develops in the sinuses and is characterized by the ample quantities of leucocytes and the admixture of desquamated endothelial structures, bacilli, and all sorts of detritus, as dead cells and bacteria. In some cases we have found this material to be ingested by the macrophages which occur in great numbers.

In the tunica propria of the *trachea* there are hyperaemia, oedema and haemorrhages—visible already to the naked eye—and in some cases also moderate numbers of granulocytes.

The *lungs* in each case show interstitial infiltrations of pseudo-eosinophiles and eosinophiles, either in a diffuse manner both in the



Figs 1 2

Fig 1 O 5397 59 Rabb t k dnev Hematoxyl n eos n 300 × Bacterial masses in the glomerular space with heavy compressi n of the tuft
Fig 2 P 238 60 Rabb t ki lney Hematoxylin e sin 300 × D fferent types of tubular derangement Strongly ac d phil casts



Fig 3

a) and b) O 5396 59 Rabbit kidney I ade g 125 × c) P 238 60 Rabb t k dney Hematoxyl n e s n 125 × Note the pre ence in the glomerular space an l in the tul ules of str ngl ac d ph l casts and the l fferent types of tubular degenerati n

interalveolar and in the peribronchial and perivascular connective tissue or as more circumscribed infiltrations particularly in the inter lobular interstice The vessels on the other hand show an obvious correspon ling granulocytosis and it is often possible to follow the very migration of the granulocytes even through arterial walls of con sideral le thickness surrounded by particularly ample infiltrations In addition to the infiltrations of granulocytes the material generally also shows a rather d stinct hyperplasia of the peribronchial lymph nodes Emphysema at lectasis and oedema are found in places and in some minor sections there are signs of an acute serous pneumonia with great numl ers of granulocytes in the exudate and also some desquamated epithelia The exudate as well as the oedema in many cases is strongly acidophil obviously a result of haemolysis



Fig 4

O 5403/59 Rabbit liver Hematoxylin eosin 300 × Pronounced vacuolization of the liver cells in the centro-lobular area The vacuoles are histologically lipid free

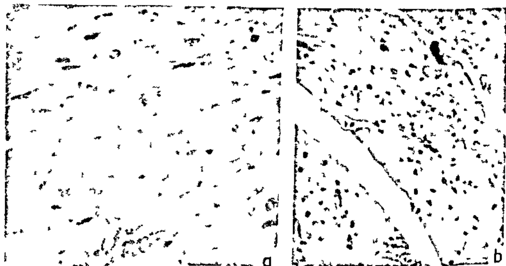


Fig 5

O 5402/59 Rabbit myocardium Hematoxylin eosin a) 500 × b) 300 × The pictures show different types of myocadial degeneration In a) there is a granular decomposition (cloudy swelling) whereas in b) there are homogenized and highly *acidophil muscle fibres showing pyknosis*

The *liver* also contains considerable numbers of granulocytes both in the sinusoids and in the periportal connective tissue In each case the liver cells in the centro-lobular areas show signs of vacuolization The vacuoles may reach the size of nuclei, and they are not Scarlet-positive There are generally two or more vacuoles in each cell which, in fact, is often so swollen that the trabecular structure has disappeared completely In these cases it is often possible to demonstrate nuclear changes as pyknosis and karyolysis Located centro-lobularly the areas containing these vacuolized liver cells may show great irregularities,

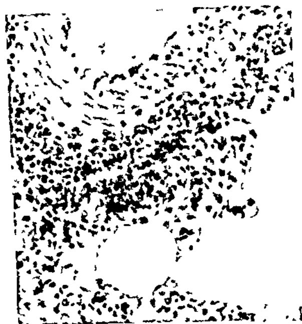


Fig 6

O 5398 59 Rabbit lung Hematoxylin eosin 300 × Heavy accumulation of pseudo eosinophiles and eosinophiles perivascularly

may be attached eccentrically to the central vein, and may incorporate a sector of varying size

The *myocardium* often shows signs of a degenerative process in the form of patches of uneven stainability of the muscle fibres, disappearance of the striation, and sometimes karyolysis In other cases the sarcoplasm becomes the site of a granular, scaly or discoidal decomposition, sometimes even associated with sarcolysis In some sections there may also be areas comprising swollen, homogenized, highly acidophil muscle fibres showing pyknosis There has been no fatty infiltration in our material

In the *kidneys* we have found extensive damage to various parts of the nephron the changes sometimes having a mainly proximal, sometimes a more distal site In each section the changes may include most of the nephrons, though with certain patches more strongly marked As for the glomeruli there are considerable quantities of bacilli in the glomerular space, accompanied by compression atrophy, sometimes even necrosis of the glomerulus In some cases the glomerular space is filled with a highly acidophil liquid with or without red cells The latter show a distinct poikilocytosis The proximal and distal convoluted tubules and the loop of Henle, as already mentioned, show different degenerative phenomena in different parts, either with a distinctly

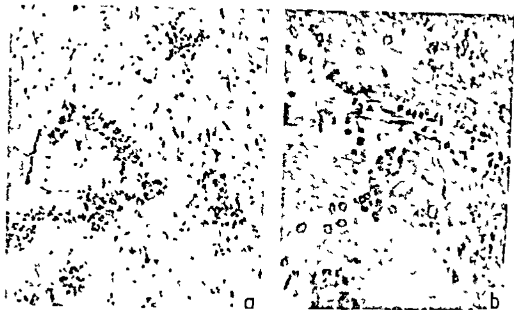


Fig 7

P 237/60 Rabbit brain Hematoxylin eosin a) 125 × b) 300 × Perivascular and pericellular basophil bodies with a somewhat grape like appearance

acidophil, homogeneous plasm and karyopyknosis or with a swollen, vacuolized "foamy" plasm and an apparently normal nucleus Furthermore, the cytoplasm often contains "hyaline" granules of various sizes, the lumen containing "casts" comprising either a markedly acidophil homogeneous mass or elements of a corpuscular structure, as red blood cells in various phases of decomposition, other cellular débris, and bacteria In certain cases most of the collecting tubules may contain "casts" of this kind, generally of the homogeneous type The contents of the glomerular space and tubuli and the "hyaline" intraepithelial granules are positive to benzidine and assume an attractive vermilion colour with Ladewig's stain They are negative to PAS staining and to Alcian Blue and show no metachromasy with Toluidine Blue Finally, the kidneys show signs of a marked anemia

The *suprarenal glands* in a few cases showed small haemorrhages and cortical necrosis The latter generally displayed a moderate granulocytic cellular infiltration The lipid content in the cortex varied but was generally rather high, in particular in the zona fasciculata

In the white substance in various portions of the *brain stem* we have found peculiar basophil structures, slightly granular, sometimes spherical, sometimes cloudy, and showing a perivascular or pericellular arrangement at various levels These structures are distinctly PAS positive and show metachromasy with Toluidine Blue at pH 3 6 6 1

The other organs subjected to microscopical examination, as the stomach and intestinal canal, revealed nothing noteworthy in addition to the naked-eye appearances

DISCUSSION

In the present treatise on the pathophysiology of experimental anthrax in rabbits our interest has centred in particular on the terminal course of the disease. In this phase of the infection there are obvious changes in the physiology of respiration as can also be understood from the post mortem appearances.

These changes in the field of respiration physiology—described by us already in 1939—may be characterized as an asphyxia associated with an extremely low O_2 concentration in the blood and an advanced haemolysis.

The O_2 concentration in the blood has proved to be exceptionally low in the pre-agonal stage of the process. Applying the technique of *van Slyke* the O_2 content of the arterial blood is found to be 0.1–0.2 per cent (by volume) 5–10 min. before death. This very low figure seems to be typical of the terminal stage of anthrax as can be seen from a comparative investigation of the pre-agonal stage in infectious disease caused by Pasteurella multocida and Streptococcus pyogenes. Although there is a drop in blood O_2 even here, it is moderate as compared with the values found in anthrax and seldom reaches below 50 per cent of the initial normal O_2 concentration. Not even in strangulation of anaesthetised rabbits was it possible to produce so low O_2 concentrations in the arterial blood as are found in the terminal stage of anthrax. Injections of Clostridium novyi toxin in rabbits do not cause such a strong reduction of the O_2 content as B. anthracis.

It has also been possible to demonstrate *in vitro* the ability of B. anthracis to reduce the O_2 concentration in blood. In normal blood infected with a culture of B. anthracis and incubated for 6 hr. at 37° C there is a considerable decrease in blood O_2. The same procedure carried out at 4° C does not result in any change in the O_2.

Even if the concentration of O_2 in the blood is very low during the terminal stage of anthrax the oxygen capacity of the haemoglobin is obviously not noticeably impaired. When dark anthrax blood poor in O_2 is exposed to the air in a glass flask the colour changes to the light red typical of HbO_2. Consecutive blood gas analyses of the blood thus aerated show, however, that the O_2 content returns to an extremely low value already within 1–2 hr.

The question is whether the low O_2 content in the blood can be attributed to the O_2 consumption by the anthrax bacillus. There are two facts speaking against such an interpretation. (1) The CO_2 content in blood from anthrax infected rabbits is below normal in the terminal stage and this should not be the case if the bacteria themselves had a high O_2 consumption with an ensuing release of CO_2. (2) Our investigations applying the *Warburg* technique have demonstrated that even if the O_2 consumption of B. anthracis must be considered rather big it does not differ much from the O_2 consumption of other bacteria.

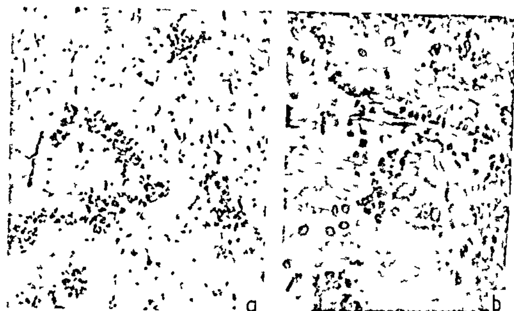


Fig 7
P 237/60 Rabbit brain Hematoxylin eosin a) 125 × b) 300 × Perivascular and pericellular basophil bodies with a somewhat grape-like appearance

acidophil, homogeneous plasm and karyopyknosis or with a swollen vacuolized "foamy" plasm and an apparently normal nucleus Further more, the cytoplasm often contains "hyaline" granules of various sizes the lumen containing "casts" comprising either a markedly acidophil homogeneous mass or elements of a corpuscular structure, as red blood cells in various phases of decomposition, other cellular débris, and bacteria In certain cases most of the collecting tubules may contain "casts" of this kind, generally of the homogeneous type The contents of the glomerular space and tubuli and the "hyaline" intraepithelial granules are positive to benzidine and assume an attractive vermilion colour with Ladewig's stain They are negative to PAS staining and to Alcian Blue and show no metachromasy with Toluidine Blue Finally, the kidneys show signs of a marked anemia

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In the white substance in various portions of the *brain stem* we have found peculiar basophil structures, slightly granular, sometimes spherical, sometimes cloudy, and showing a perivascular or pericellular arrangement at various levels These structures are distinctly PAS positive and show metachromasy with Toluidine Blue at pH 3 6 6 1

The other organs subjected to microscopical examination, as the stomach and intestinal canal, revealed nothing noteworthy in addition to the naked-eye appearances

the rabbit We also believe that the further development of the renal lesion together with the hepatic and myocardial changes is such can be explained by a marked hypoxaemic state

Finally we should like to mention the observations regarding the perivascular and pericellular precipitations of muco polysaccharides in the white matter of the brain We consider it too early for an interpretation of these findings that in certain respects show a resemblance to the mucoid degeneration of oligodendrocytes but in other respects give the impression of artifacts

SUMMARY

The authors publish the result of certain pathophysiological investigations of experimental anthrax in the rabbit Clinically, the terminal stage of the disease is characterized by asphyxia associated with an extremely low O content in the blood (Tables 1 and 2) The O_2 content has been studied according to the technique of van Slyke In the arterial blood it was found to average 0 2 per cent by volume prior to death In tests using other infective agents as Pasteurella multocida Streptococcus pyogenes and toxin from Clostridium novyi the O concentration prior to death has been 9 6 10 1 and 5 6 per cent by volume respectively (Tables 3 5) In cases of mechanically induced asphyxia the ante mortem O concentration was 6 1 per cent by volume (Table 6)

The unusually low O concentration is not a result of the consumption of gaseous oxygen by B anthracis (as determined according to Warburg) nor does it follow from the bacterial interference with the metabolism of the individual organs as has been shown by incubating liver slices together with cultures of B anthracis

When the temperature curve has reached a maximum in cases of experimental anthrax in the rabbit there is a reduction in the number of erythrocytes and in the haematocrit value (Table 9) This is *not the* case however in experimental infections by Pasteurella multocida and *toxin of* Clostridium novyi in the rabbit (Table 10) In the terminal stage of anthrax there is a reduction of the osmotic resistance of the red corpuscles (Table 11)

Apart from symptoms of sepsis as splenitis lymphadenitis and petechial haemorrhage the findings of pathologic *anatomical interest* have been a renal lesion of the haemoglobinuric nephrosis type hepatic and myocardial degeneration phenomena probably of hypoxaemic origin and further perivascular and pericellular precipitations of muco-polysaccharides in the white matter of the brain resembling the mucinoid degeneration of oligodendrocytes (artifacts?)

Authors conclude that the terminal stage of experimental anthrax in rabbits is dominated by an *extremely* low concentration of O in the blood and that the animals actually succumb to asphyxia Although not the only cause the haemolysis which has been shown is one of the factors responsible for this asphyxia

Another question of interest in this connexion is whether B anthracis exerts a general blocking effect on cellular respiration This is not the case however as can be found from our experiments in which liver slices were incubated together with anthrax cultures according to the *Warburg technique It was not possible to find any influence of the* bacteria on the respiration of the liver cells

An investigation of the functional state of the erythrocytes in the terminal stage of anthrax reveals a considerable reduction of the osmotic resistance which explains the advanced haemolysis known to exist in the latter part of the course of experimental anthrax This haemolysis naturally plays a part in the reduction of blood O_2 but cannot offer the whole explanation of the low O_2 values in cases of anthrax The problem whether the low O_2 content in anthrax blood can be sufficiently explained by *this haemolysis has to be left unsettled for* the time being

Considering all the various findings reported in this article it can be stated that the terminal course of anthrax is dominated by an extremely low O_2 content in the blood causing the death of the animals from asphyxia The haemolysis shown to be present is merely one contributory cause of the asphyxia the basic cause of the extremely low O_2 values being unknown at present

Apart from signs of sepsis as splenitis lymphadenitis and petechial haemorrhage the morbid anatomy findings indicate (a) renal damage of the haemoglobinuric nephrosis type (b) certain extensive degenerative changes in the liver and myocardium and (c) the presence within the white matter of the brain of a perivascular and pericellular substance which judging from its staining reactions seems to be composed of acid muco polysaccharides *The post mortem appearances indicate* a massive rapid haemolysis associated with the development of a renal lesion caused in part directly by the local effects of haemoglobin in part probably by renal ischaemia The latter condition may be part of a symptom complex indicative of general hypoxaemia or it may have its origin in a state of shock accompanied by contraction of the renal arteries The centro lobular liver cell degeneration is of a type which since the investigations by *Buchner* (1942) *Altmann* (1949) and others is believed to be a sequela of hypoxaemic conditions Even the myocardial lesion may be interpreted in the same way The renal changes observed by us probably coincide in the main with the changes reported by *Ross* and *Cemansky* from a material of guinea pigs and mice respectively These authors however have preferred not to relate the renal damage directly to haemolysis *Ross* considers the lesion to be a *lower nephron nephrosis i e* a haemoglobinuric nephrosis however without giving any detailed picture of the massive decomposition of tissue of some kind which is generally found underlying such lesions

In our opinion the renal lesion can support the theory of haemolysis is one factor though not the only one in experimental anthrax in

BRIEF REPORTS

A SIMPLIFIED METHOD FOR MAKING CHROMOSOME PREPARATIONS FROM SKIN BIOPSIES

By *Anders Frøland*

The aim of the present report is to describe a method for chromosome preparation from skin biopsies in which the methods of *Harnden* (1960) for the tissue culture and the techniques of *Moorhead et al* (1960) for the preparation of slides are combined Our method closely follows the original schedule outlined by *Rothfels* & *Siminovitch* (1958) for the study of chromosomes in monkey cells

Specimens are obtained by means of the technique described by *Edwards* (1960) which in our opinion causes very little pain and leaves only a minute scar

The specimen is cut into very small pieces (½ mm × ½ mm) and fixed to the bottom of a 25 cc Erlenmeyer flask with a reasonably flat bottom (6–8 explants in each) in a plasma clot consisting of cockerel plasma and chick embryonic extract 4 cc of a nutrient composed of 70 per cent medium 199 (Glaxo) 20 per cent pooled human plasma and 10 per cent embryonic extract is added 25–50 per cent of the

brought into suspension

New culture flask are set up each containing 4 cc of the nutritient mixture inoculated with the cell suspension to give a final concentration of approximately 5000 cells per cc

In about a week the cells will have grown out to cover the bottom of the flask Colcemid (Ciba)* is added to a final concentration of 0 004 per cent and left to act for 20 hours

to
a
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sl[illegible] warm stream of air onto them or by fanning above a spirit lamp Finally the preparations are stained with aceto orcein and mounted in Euparal

Results and Discussion

This technique which is a combination of two wellknown methods seems to give preparations with an appreciable amount of well spread mitoses suited for chromosome analysis

The chromosomes do not become unduely contracted although the colcemid is left act for 20 hours

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Received 11 x 61 *from the University Institute for Human Genetics (Tage Kemp M D) Department of Experimental Genetics and Cytology (J Schult Larsen M D)*

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* Generously supplied to us by Ciba Farver & Farmaceutika A/S Copenhagen

The expert technical assistance of Miss Birgit Pedersen and Miss Kirsten Niss Pedersen is gratefully acknowledged

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A SIMPLIFIED METHOD FOR MAKING CHROMOSOME PREPARATIONS FROM SKIN BIOPSIES

By *Anders Frøland*

The aim of the present report is to describe a method for chromosome preparation from skin biopsies, in which the methods of *Harnden* (1960) for the tissue culture and the techniques of *Moorhead et al* (1960) for the preparation of slides are combined Our method closely follows the original schedule outlined by *Rothfels & Siminovitch* (1958) for the study of chromosomes in monkey cells

Specimens are obtained by means of the technique described by *Edwards* (1960), which in our opinion causes very little pain and leaves only a minute scar

The specimen is cut into very small pieces (½ mm × ½ mm) and fixed to the bottom of a 25 cc Erlenmeyer flask with a reasonably flat bottom (6–8 explants in each) in a plasma clot consisting of cockerel plasma and chick embryonic extract 4 cc of a nutrient composed of 70 per cent medium 199 (Glaxo), 20 per cent pooled human plasma and 10 per cent embryonic extract is added 25–50 per cent of the n

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brought into suspension

New culture flask are set up each containing 4 cc of the nutritient mixture inoculated with the cell suspension to give a final concentration of approximately 5000 cells per cc

In about a week the cells will have grown out to cover the bottom of the flask Colcemid (Ciba)* is added to a final concentration of 0 004 per cent and left to act for 20 hours

The cells are then trans

to test tubes

fixative) on

slowly by b ... slightly warm stream of air onto them or by fanning above a spirit lamp Finally the preparations are stained with aceto orcein and mounted in Euparal

Results and Discussion

This technique, which is a combination of two wellknown methods, seems to give preparations with an appreciable amount of well spread mitoses suited for chromosome analysis

The chromosomes do not become unduely contracted, although the colcemid is left act for 20 hours

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In the author's experience it offers the advantages of including only one subculture of the cells and in avoiding the delicate handling of coverglasses. Moreover in a cytogenetic laboratory, where routine chromosome preparations are made from blood cultures much time and labour is saved because the preparation of slides from the skin cultures can be made alongside those from the blood cultures.

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FROM THE DEPARTMENT OF ANATOMY UNIVERSITY OF HELSINGFORS (HEAD GÖRAN HJELMMAN M.D.)

THE MITOTIC REGENERATION OF MAST CELLS IN THE LOWER RESPIRATORY TRACT OF HUMAN EMBRYOS

By

BERTEL GRAHNE

Received 19 ii 61

The question of the regeneration of the mast cells is not fully understood It has been assumed that both homoplastic and heteroplastic regeneration takes place

The data in the literature regarding the occurrence of mitoses in the mast cells are few, however In the spleen of a lizard species (gongyrus) *Michels & Nelis* (1922) observed mitotic mast cells in large numbers *Lehner* (1924) mentioned that he had seen mitoses in mast cells in the mucous membrane of the sphenoid cavity and in the epiglottis in man and in the pyloric mucosa in mice *Alfejew* (1924) reported that she had observed mast cell mitoses in mouse embryos measuring 16 mm, in guinea pig embryos measuring 48 mm in rabbit embryos measuring 55 mm and in newborn cats She suggested that an abundant granulation in the mast cells may hide or obscure the mitoses possibly present Hence she assumed that mitoses could only be distinguished in sparsely granulated mast cells *Hauser* (1937) stated that he had detected only a few mast cell mitoses in the parotid in ruminants *Paff, Bloom & Reilly* (1947) observed mast cell mitoses in the tissue culture from a mast cell tumour from a dog *Zifcer Elsasser & Kirk* (193[illegible]

[illegible] mast cells in metastases of sarcoma Furthermore *Hirschmann* (1919) who studied the mast cells in the larynx in chronic laryngitis of non specific pathogenesis *Higuchi* (1930) who investigated the mast cells in the mammae, and *Drennan* (1951) who investigated the mast cells in albino rats reported that they had not been able to detect any mast cell mitoses

MATERIAL AND METHODS

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granules (*Holmgren*

The material consisted of 30 human embryos with a crown rump length of 3 1 to 35 cm, the difference in length between the various embryos was about 1 cm Specimens for investigation were taken from the following seven sites the larynx so as to include a portion of the ventricular cord the vocal cord and the laryngeal ventricle, the upper trachea, the lower trachea, the two main bronchi, the hyparterial lobe bronchi to the right and left From the smallest eight embryos with a crown rump length varying between 3 1 and 10 9 cm specimens could not be obtained from all seven regions owing to the minute size and low degree of development

The pieces of tissue were fixed in a freshly made up 4 per cent basic lead acetate solution embedded in paraffin and sectioned at 10 µ Staining was performed in a 1 per cent aqueous solution of toluidine blue for 10–15 minutes The mast cells in the subepithelial layer were studied

RESULTS

In the smallest twelve embryos, with a crown-rump length of 3 1 to 15 3 cm, no mast cell mitoses were discernible The smallest embryo in which mitoses were observed measured 16 cm In the embryo next in size, with a crown rump length of 17 cm, mitoses were found in the mast cells in all seven regions investigated, although in the larynx and trachea they were few in number In the next two embryos, measuring 18 and 19 3 cm, a few mitoses were observed in the mast cells in the lobe bronchi, but none in the other regions In the remaining embryos mitoses were detected only in one, which measured 23 5 cm This embryo exhibited a few mitotic mast cells in the larynx

DISCUSSION

It was an interesting observation that the number of granules per mast cell was small in the small embryos, and that granulation increased with the degree of development It is possible that *Alfejew* (1924) was right in her assumption that the presence of mitoses may be obscured by a rich granulation, and that this was the case in regard to the largest embryos in the present investigation No mitoses were discernible in the twelve embryos, however, which measured less than 16 cm, although the granulation in their mast cells was so scanty that it could not have been obscured by the presence of mitoses Furthermore, in the embryos with a crown-rump length exceeding that of the embryos in which mitoses were observed, the amount of granulation did not increase so rapidly that the observation of mitoses would suddenly have become impossible The granulation in the mast cells prevented exact counting of the various mitotic figures, however, and therefore less exact expressions for the determination of the number of mitoses have been used In any case the occurrence of mitoses seems to show that some mast cells, at least, undergo mitotic division during certain developmental stages

SUMMARY

The mast cells were investigated in seven regions of the subepithelial tissue in the larynx, trachea and bronchi in 30 human embryos with a

crown rump length varying between 3 1 and 35 cm Mitoses were observable in some mast cells in the regions investigated in five embryos with a crown rump length varying between 16 and 23 5 cm The presence of mitoses seems to show that mitotic division of the mast cells is possible during certain developmental stages, at least

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Pal C D n

(FROM THE DEPARTMENT OF PATHOLOGY, SECTION II, UNIVERSITY OF HELSINKI HEAD HARALD TEIR M D)

RESPONSE OF TISSUE EOSINOPHIL LEUCOCYTES IN THE DUODENAL LAMINA PROPRIA TO INTESTINAL OBSTRUCTIONS APPLIED

By

BORJE SUNDELL and GUSTAF FOCK

Received 9 x 61

The number of eosinophil leukocytes in the blood decreases on the day of operation (5), which has been interpreted as an expression of the emotional stress that causes an increased level of 17-ketosteroids and 17-hydroxycorticosteroids and also brings about an increased concentration of 18 hydroxycorticosteroids in the plasma

In earlier experiments made in our laboratory the eosinophil leukocyte has been studied in tissues after influence of various pharmaca and corticosteroids (9, 10, 11) Further, *Rytomaa* (6) has investigated the distribution of eosinophil leukocytes in different organs and correlated these observations with the eosinophil leukocytes of the blood For this reason we thought that it might be of interest to study the relation in connecion with applied obstruction in various portions of the intestinal loop

MATERIAL AND METHODS

For the experiments 2 to 4 months old female rats of the Sprague Dawley strain were used Their weight varied from 200 to 150 grams In the course of the experiments all animals were housed under identical conditions They were fed from the kitchen of the surgical clinics of the Central Hospital of the University of Helsinki All animals were killed at the same time of the day to obviate diurnal variations in the number of eosinophil leukocytes that have been found to occur in the blood of rat (4) and mouse (3)

Operative measures The intestinal obstruction was applied in ether narcosis of 5 to 10 minutes duration The abdominal skin was shaved and dried with a 2 per cent solution of chloramine The abdomen was incised in the median line The obstruction was applied with a thin silk ligature arund the intestinal wall without damaging the blood vessels of the intestine or the serosa After that the abdomen was closed with a continuous silk suture

Histological preparations The animals were decapitated without anaesthetics Tissue specimens were removed from the small intestine 2 cms aborally to the pyloric valve and were fixed immediately in Bouin s solution After embedding of the preparations in paraffin histological sections of 3 μ were cut which were stained with hemalum eosin

The eosinophil count The eosinophil count was obtained by counting the number of cells in an effective area of the mucosa of the small intestine We obtained the lamina propria of the small intestine as 2o 7 per cent of the mucosa A microscope with a 10 × planocular 90 × apochromatic objective and Zeiss micrometer eye piece was used

Mean values were calculated and the standard error of the means was obtained from the formula $S_{\bar{x}} = \frac{(x - \bar{x})^2}{n(n-1)}$ where x indicates a single observation, $\bar{x}$ the mean value and n the number of observations. The significance of difference was calculated with the use of the "Student's" t test as described by Snedecor (1946) (8). The P values were obtained from the tables of *Fisher & Yates* (1933) (2). A difference was considered highly significant when $P < 0.001$, significant when $P < 0.01$ and probably significant when $P < 0.05$.

RESULTS

Response to jejunal obstruction. This group includes 20 experimental animals. As early as the first day after the application of the intestinal obstruction the condition of the animals was poor. The intestines were largely expanded above the point of obstruction.

The histological preparations displayed accumulation of inflammatory cells in the lamina propria in so large amounts that to count the eosinophil leukocytes was out of the question. This group was excluded. Animals which survived the first 24 hours did better, in spite of the fact that the intestinal obstruction was found to be complete. An insignificant decrease of the number of eosinophil leukocytes was noticed in comparison with normal animals.

Response to obstruction in the terminal ileum. This experiment includes 30 animals which better stood the intestinal obstruction. The number of animals is distributed evenly on different days (5 animals). Up to the 3rd postoperative day the animals exhibited moderately expanded intestines and a rather good general condition. Histologically this period of normal and subnormal values corresponds to the eosinophil leukocytes in the lamina propria. On and from the 4th postoperative day the intestine was more expanded and the general condition of the animals was aggravated. The number of eosinophil leukocytes moderately increases, however, not significantly in comparison with normal values. After this day a gradual decrease in the number of eosinophil leukocytes occurs until the lowest value was reached on the 7th day when the animals were in agone. Significance is reached between the values of the 4th and 7th day.

Response to obstruction in the ascendent colon. In this group 30 experimental animals were used and they are distributed evenly on the various days. Up to the 5th postoperative day the animals exhibited moderately expanded intestinal loops. The number of eosinophil leukocytes which were normal on the 2nd postoperative day, steadily increased up to the 5th day, when significance was reached. After that the number substantially falls so that a high significance is reached as compared with the values of the 5th postoperative day. On the 7th day an increase in the number of eosinophil leukocytes was ob[illegible] more. Th[illegible]

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30 exp[illegible] [illegible] early as the 2nd day an increased number

of eosinophil leukocytes was counted in the lamina propria. Significance was not obtained in comparison with normal animals. During the following postoperative days the number of eosinophil leukocytes falls so that normal level is reached on the 6th postoperative day and highly significant low values on the 7th day. To these responses clinically corresponds very expanded *intestinal loops on and from the 5th day*. The animals were in general in good condition despite intestinal obstruction of one week's duration.

Response to ether anaesthesia and starving. This control group includes 20 animals. The first 24 hours a clear significance in the number of eosinophil leukocytes in comparison with normal values was found. Already on the 2nd day the number of eosinophil leukocytes falls to values that are not significant. Nor are these values significant in comparison with other groups. After a transient increase on the 3rd day the number of eosinophil leukocytes once more approximates values that correspond to other groups on the 4th day.

TABLE 1

Number of Tissue Eosinophils in the Duodenal Lamina Propria Influenced by Experimental Intestinal Obstructions

Intestinal obstruction	Duration of obstruction	Number of animals	Average number of tissue eosinophils ± standard error	Probability P
Jejunal	2 days	5	364 ± 59	
Terminal ileum	2 days	5	331 + 46	
	3	5	294 ± 45	
	4	5	520 ± 60	
	6	5	276 + 49	0.01
	6	5	288 ± 47	
	7	5	82 + 47	
Ascendent colon	2 days	5	311 + 30	
	3	5	510 ± 122	
	4	5	576 ± 161	
	5	5	729 ± 54	0.001
	6	5	202 ± 43	
	7	5	485 + 63	
Descendent colon	2 days	5	572 ± 54	
	3	5	502 ± 111	
	4	5	424 + 12	
	5	5	372 ± 10	0.001
	6	5	316 ± 93	
	7	5	137 + 40	
Starving and ether anesthesia	1 day	5	1010 + 35	
	2 days	5	683 + 21	
	3	5	855 + 98	
	4	5	648 + 139	
Controls		14	405 + 92	

DISCUSSION

An intestinal obstruction is a severe disorder afflicting the organism The higher up in the intestines the obstruction is located, to higher degree is the organism affected This fact was reflected also in these animal experiments, in which rats with jejunal obstruction did not survive the second day, while animals with the obstruction in the terminal ileum in some cases got on for as long as a week Obstructions in the colon did not affect the experimental animals at the beginning, and they exhibited severer symptoms only at the end of the first week

These changes in the general condition could be followed by the responses of the eosinophil leukocyte in the duodenal lamina propria After the shock like effect of the 2nd day, when the number of eosinophil leukocytes was fairly low, or corresponded to normal values, the number significantly increased in the period 2nd to 4th day In this period the condition of the experimental animals was the very best The increased number of eosinophil leukocytes might be interpreted as a defensive measure against both the contents accumulated in the intestines and toxaemia due to necrosis of tissue During this period corticosteroids are being secreted, which also causes tissue eosinophilia to arise, of which there is experimental evidence (9, 19 11) Whether this tissue eosinophilia is produced through diapedesis from the blood into lamina propria or by way of neoeosinopoiesis in the lamina propria itself, is wrapped in obscurity as yet According to Rytomaa (6) the blood is well capable of discharging the eosinophil leukocytes that would be conditioned by the eosinophilia in the lamina propria

After the 5th day the number of eosinophil leukocytes decreases rather rapidly, which might be interpreted as a sign of the organism being exhausted It has further been shown by experiments that tissue eosinophilia is transformed into eosinopenia after repeated injections of cortisone to experimental animals (7, 9) This period also coincides with the tissue phase of the eosinophil leukocytes so as it is assumed and calculated by Rytomaa (6) The tissue phase was calculated as approximately 4 days and in earlier experiments it was assumed to be 5 to 6 days (1) After this period the cells are destroyed, or are possibly returned into the circulation In these cases of intestinal obstruction a destruction of the cells might be the most probable

Quite a similar behaviour pattern is seen in the blood of man in connection with operative measures (5)

A comparative study of blood—tissue in all probability would give interesting insight into what happens to the eosinophil leukocyte in an intestinal obstruction

SUMMARY

The study comprises a total of 144 experimental animals of the Sprague Dawley strain It attempts at elucidating the manner of re

of eosinophil leukocytes was counted in the lamina propria. Significance was not obtained in comparison with normal animals. During the following postoperative days the number of eosinophil leukocytes falls so that normal level is reached on the 6th postoperative day and highly significant low values on the 7th day. To these responses clinically corresponds very expanded intestinal loops on and from the 5th day. The animals were in general in good condition despite intestinal obstruction of one week's duration.

Response to ether anaesthesia and starving. This control group includes 20 animals. The first 24 hours a clear significance in the number of eosinophil leukocytes in comparison with normal values was found. Already on the 2nd day the number of eosinophil leukocytes falls to values that are not significant. Nor are these values significant in comparison with other groups. After a transient increase on the 3rd day the number of eosinophil leukocytes once more approximates values that correspond to other groups on the 4th day.

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Controls		14	405 ± 92	

THE EXPERIMENTAL PRODUCTION OF HEPATIC VENO OCCLUSIVE DISEASE IN RATS

By

Cynthia F Bruun Hanssen

Received 10 iv 61

In a preliminary communication to The Lancet from this department (6) it was stated that veno occlusive disease had been produced in rats by the injection of the pyrrolizidine alkaloid monocrotaline ($C_{16}H_{23}O_6N$) obtained from several species of Crotalaria This communication describes the experiments which have been performed to further this study

Human veno occlusive disease has been encountered in the West Indies and the essential pathology (2) was said to be a connective tissue proliferation within the centrolobular veins with partial or complete occlusion and consequent damming back of blood within the sinusoids The end result was hepatic cirrhosis with a predominantly centrolobular distribution of the fibrosis

Similar pathological changes affecting the smaller tributaries of the hepatic vein have been found or described in various naturally occurring diseases of animals Such diseases are the Walking Disease of horses and cattle in South Africa and ragwort poisoning in the United Kingdom (5) The above diseases in animals are said to be due to senecio poisoning and there is some evidence that the human disease is also caused by the ingestion of senecio compounds (10) which are used to make bush teas (1 5)

Senecio poisoning is caused by the pyrrolizidine group of alkaloids which are found in plants of the genus *Senecio* (Compositae) *Crotalaria* (Leguminosae) and *Heliotropium* (Boraginacea), (5)

EXPERIMENTAL

The animals used were Wistar albino rats aged 7 to 8 weeks and of weight 100 g Th —
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sponse of the eosinophil leukocyte in the wall of the duodenal intestine in connection with a mechanical intestinal obstruction

The intestinal obstruction was operatively applied by way of a silk suture in the jejunal loop, the terminal ileum, the ascendent colon, the transverse colon and the sigmoid colon

High intestinal obstruction (jejunal) on the 2nd day caused the animals' death, whereas the obstruction in other portions of the intestine could be endured up to a week

These periods corresponded to an initial decrease and low values of eosinophil leukocytes in the lamina propria Thereafter a gradual increase occurred up to the 4th and 5th days After that the number of eosinophil leukocytes decreased until the animals on the 7th day were in agone

These responses are interpreted as being dependent, to begin with, on a shock-like effect, after which the animal's defensive forces bring about eosinophilia in the lamina propria, as a defence against toxins amongst others The subsequent eosinopenia is interpreted as being dependent partly on exhaustion, partly on continuous secretion of the corticosteroids, partly on the tissue phase assumed for the eosinophil leukocyte

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Fig 2
Rat liver 8 days after injection of monocrotaline showing marked dilatation of the sinusoids The centrolobular vein is partially occluded by a fine loose connective tissue network (Picro Mallory × 250)

was observed Sixty two showed other and probably associated pathological changes in the liver and only 3 showed no obvious microscopical change other than some congestion

In the centrolobular veins a variety of changes were observed including a connective tissue network obstructing the lumen similar to that described in the human V O D (9) This network appeared as fine fibrils staining blue in picro mallory preparations and light brown with silver stains Sometimes occlusion was complete and other times a narrow lumen persisted (Figs 2 3 and 4)

Many of the veins were lined by moderately large oval or polyhedral cells with a single central nucleus and similar cells were lying free within the lumina Oft times these cells appeared in two or three layers lining the wall or were so numerous within the lumen as to form a cellular obstruction within the vein (Fig 3) Considerable amounts of fibrin which appeared to be undergoing organisation were present in some veins A fibrinous exudate surrounded many of the veins and a similar exudate could be seen in the interlobar fissures and extracapsularly (Fig 1)

In all the livers there was sinusoidal congestion frequently with gross distension so that the appearance was often one of blood filled lagoons (Fig 2) Centrolobular haemorrhages and necrosis were present in nearly every case In haematoxylin and eosin preparations it was sel



Fig 1

Rat liver 4 days after injection of monocrotaline showing marked centrolobular necrosis and a fibrinous exudate surrounding the veins (Picro Mallory × 40)

to make a 2 per cent strength The control animals were given intraperitoneal injections of N/10 hydrochloric acid and water in the appropriate amounts In these experiments all the animals were fed on normal diet of laboratory chow (Diet No 41, Oxo) The liver speciments were fixed in formalin and stained by the usual routine procedures

RESULTS

A total of 70 animals were injected with monocrotaline and 67 of these were available for post mortem study Except for one rat which was killed after 27 days, all animals died or were killed because of sickness in 2 to 10 days after the first injection Twenty-five control rats were used

Of the 67 rats examined macroscopically, 58 showed marked ascites but only 10 appeared jaundiced Eleven rats showed small haemorrhages in other viscera than the liver

The macroscopic appearance of the liver was similar in every case The livers were congested and showed a fine red-yellow mottling on the external surface The cut surface was similarly mottled, and this was even more clearly defined after fixation The appearances were similar to that of venous congestion of the liver as seen in cardiac failure in humans The control series of rats were not remarkable

Of the 67 rats examined after death, 65 livers were suitable for histology, and in 28 (42 per cent) of these some degree of venous occlusion



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Rat l ver 8 days after nje t on of monocrotal ne shov ng marked dilatation of the s nuso ds The centrolobular ve n s part ally occluded by a f ne lo se connective t ssue network (P cro Mallory × 250)

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In all the livers there was sinusoidal congestion frequently with gross distension so that the appearance was often one of blood filled lagoons (Fig 2) Centrolobular haemorrhages and necrosis were present in nearly every case In haematoxylin and eosin preparations it was sel



Fig 3
Rat liver 10 days after injection of monocrotaline showing partial occlusion of the centrolobular vein due to "endothelial proliferation" (H and E × 250)

dom possible to identify veins within the haemorrhagic areas but connective tissue stains revealed the venous walls The surviving liver cells were a little distorted but otherwise showed no constant pathological change At times the necrotic areas showed a fine connective tissue replacement in which occasional fibroblasts could be identified Cirrhosis was not observed in these short term experiments The control series of rats were not remarkable

DISCUSSION

When the histological appearances were related to the survival time after injection a constant pattern became evident Sinusoidal congestion was always present and haemorrhagic necrosis occurred in almost every case even though the survival period was only 2 days

Venous occlusive lesions were never seen early The oval and polyhedral cells lining the veins appeared about 3 days after injection, and by 6 to 7 days partial or complete obstructions were observed As the survival time was prolonged, the vascular changes were more marked Connective tissue networks, typical of the human veno-occlusive disease, were most likely to be found after 9 to 10 days' survival

These findings suggest a possible sequence of events in which the primary effect of the alkaloid is one of haemorrhagic necrosis of the liver cells and that the vascular lesions, with possible fibrin deposition

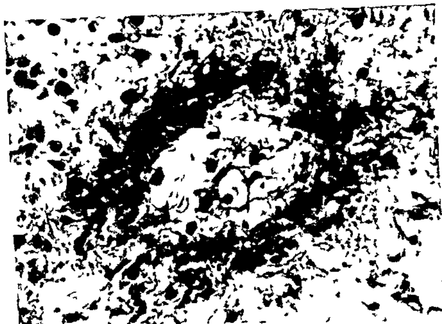


Fig 4

Rat liver 10 days after injection of monocrotaline The centrol bular vein is almost entirely occluded by a loose connective tissue in which a few polyhedral cells are enmeshed (Picro Mallory × 400)

and organisation, are secondary, this suggestion however, is not yet proven

Sinusoidal congestion is prominent and appears early, and haemorrhage may occur from rupture of the grossly distended sinusoids, the whole process in these experiments is extremely acute, and as yet no attempt has been made to study the histochemical changes in the cells which might exist before haemorrhage is evident

The nature of the polyhedral cells seen in the centrolobular veins is unknown The appearances suggest that they may be swollen endothelial cells which proliferate in response to some stimulus Some are detached and become enmeshed in fibrin within the lumen, whilst others remain in situ forming a thickened lining

If one accepts the ability of endothelial cells to change into fibroblasts then venous occlusive lesions may be produced by the endothelial cell proliferation In this Department, *Filshie & Scott* (4) have observed that endothelial swelling and proliferation can be seen in vessels at a distance from a thrombus or in experimental ligation No gross obstruction has been observed in the larger branches of the hepatic vascular tree in these rats, and histologically the endothelial proliferation was observed early and before the development of *occlusion*

The possibility of spasm occurring in the centrolobular veins cannot

be excluded It may be that the alkaloid causes vascular spasm with resultant congestion of the sinusoids and haemorrhagic necrosis, and this same spasm may initiate the endothelial changes which may lead to structural venous occlusions

Results of these tests are similar to those obtained in rats by the administration of retrorsine (3) Davidson thought that the retrorsine had a direct toxic effect on the vascular system and that endothelial proliferation was the primary lesion with haemorrhagic necrosis as a secondary change The experiments of Schoental and Head (8) using monocrotaline in rats also produced haemorrhagic centrolobular necrosis within 2 to 3 days, as did their experiments using retrorsine, isatidine and a mixture of alkaloids from S Jacobea However, the 3 latter also produced, in addition to the necrosis, endothelial proliferation of the wall of the hepatic veins (9)

SUMMARY

The characteristic changes of acute veno-occlusive disease have been produced in approximately 40 per cent of rats following the injection of large doses of monocrotaline The pathogenesis appears to be one of sinusoidal congestion and centrolobular necrosis with secondary endothelial proliferation in the veins often associated with fibrin deposition Partial or complete occlusion may consequently occur The haemorrhagic necrosis can be seen within 24 hours but veno-occlusions are not well established until 9 to 10 days after injection Further experiments have been carried out and will be reported later

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ADRENALIN INDUCED ALTERATIONS IN AORTIC TISSUE OF RABBITS COMPARED WITH CHANGES IN HUMAN AORTIC TISSUE WITH AGE

By

SV BERTELSEN

Received 27 v 61

It is generally known that several substances, injected intravenously in rabbits, may induce aortic alterations Josue (14) was the first to produce such alterations by repeated injections of adrenalin in veins The alterations are often called 'adrenalin changes" on account of the method employed in producing them The alterations can be induced not only by adrenalin and its precursor substances tyrosine and tyramine, but also by a number of other substances which vary greatly in chemical nature and biologic effect Summaries have been given by *Anitschkow* (1), *Hueper* (13), and *Raab* (23)

The object of the present paper is to report the results of a histochemical study on adrenalin-induced alterations in the aortic walls of rabbits and to compare the findings with alterations in the human aorta, reported in a previous study

MATERIALS AND METHODS

Two groups of white rabbits (weighing about 3000 g and about one year old) were kept on a uniform sufficient laboratory diet The animals in *group 1* were given intravenous injections of adrenalin in daily doses of 0 025 mg/kg until they died In *group 2* the animals were given intravenous injections of adrenalin in doses of [illegible] ys, and two days after the last injec

This publication will mention aortic alterations in a rabbit which [illegible] 3 days The aorta showed [illegible] nimal had never been in-

[illegible]

[illegible] by grants from the *Danish State Science Foundation*, and the *Danish Foundation For The Advancement Of Medical Science*

taken as serial specimens at different distances from the alterations In several cases specimens from peripheral arteries (coronary renal) were removed

The tissue was fixed in a 4 per cent formaldehyde solution and some of the specimens in 4 per cent basic lead acetate The tissue was imbedded in paraffine and cut in sections at 5 µ and stained with periodic acid Schiff s reagent (PAS) Alcian blue combined PAS Alcian blue and Hale's staining modified by Mowry, further more with hematoxylin eosin, van Gieson Hansen Eskelund s modification of Gomori s aldehydefuchsin staining and silver staining ad modum Foot The tissue fixed in lead acetate was stained with toluidine blue Lipoid was demonstrated with Sudan III, and calcium with alizarin red

RESULTS

The first gross alterations were seen as small white-gray spots in the thoracic part of aorta As the disease advances, the spots become confluent, forming large humpy depressions of a more grayish color In these areas the aortic wall is much thinner and unelastic, and these depressions may gradually bulge out, forming aneurysms with very thin, brittle and calcified walls (Fig 1)

The lesions occur most commonly in the thoracic aorta and arcus, while such changes are less frequently seen in the abdominal aorta Fig 1 shows a typical lesion in the thoracic part of aorta



Fig 1
Characteristic adrenalin induced lesions in the thoracic aorta

TABLE 1

Animals in Group 1 (Daily Injections of 0 025 mg Adrenalin per kg)

Number of Injections	Gross alterations	Microscopical pictures
4	no	normal
11	no	calcium granulets diffuse in the media
12	no	calcium granulets diffuse in the media
15	no	calcium granulets diffuse in the media
15	3 spots in the arch and a big aneurysm in the thoracic aorta	Calcium deposits (stage 2 and 3) and calcium granulets diffuse in the media
21	no	calcium granulets diffuse in the media
23	no	calcium granulets diffuse in the media

TABLE 2

Animals in Group 2 (Injection of 0 015 mg Adrenalin per kg every Second Day)

Number of injections	Gross alterations	Microscopical pictures
5	no	normal
6	no	calcium granulets diffuse in the media
7	a aneurysm in the arch and the thoracic part besides several spots in the thoracic aorta	calcium deposits (stage 2 and 3) and calcium granulets diffuse in the media
9	no	normal
11	several aneurysms in the thoracic aorta	calcium deposits (stage 2 and 3) and calcium granulets diffuse in the media
12	no	normal
13	several aneurysms in the thoracic aorta and 2 3 spots in the abdominal part	calcium deposits (stage 2 and 3) and calcium granulets diffuse in the media
13	no	calcium granulets diffuse in the media
14	several aneurysms in the thoracic aorta	calcium deposits (stage 2 and 3) and calcium granulets diffuse in the media

taken as serial specimens at different distances from the alterations In several cases specimens from peripheral arteries (coronary, renal) were removed

The tissue was fixed in a 4 per cent formaldehyde solution and some of the specimens in 4 per cent basic lead acetate The tissue was imbedded in paraffine and cut in sections at 5 μ, and stained with periodic acid Schiff's reagent (PAS) Alcian blue combined PAS Alcian blue and Hale's staining modified by Mowry, furthermore with hematoxylin eosin van Gieson Hansen Ekelund's modification of Gomori's aldehydefuchsin staining and silver staining ad modum Foot The tissue fixed in lead acetate was stained with toluidine blue Lipoid was demonstrated with Sudan III and calcium with alizarin red

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TABLE 1

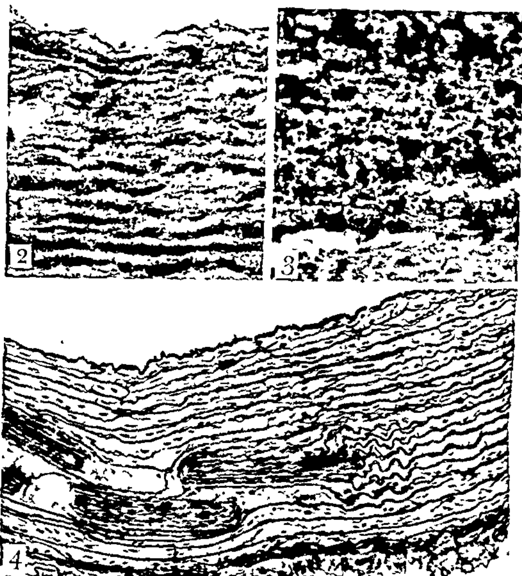
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Figs 2–4

Fig 2 (Alizarin red × 350) *Stage 1* The elastic fibres are closely packed and show a regular, undulated course Calcium granulets are precipitated among the medial *elastic fibres in the vigorously metachromatic ground substance The intima consists* of a scanty subendothelial zone of mucoid substance

Fig 3 (Alizarin-red × 525) The periphery of a calcium deposit The big calcium granulets are seen among the elastic fibres The fibroblasts and muscle cells are normal and no signs of atrophy or necrosis are seen

Fig 4 (Eskelund's modification of Gomori's aldehyde-fuchsin × 140) *Stage 2* The elastic fibres are stretched out and packed more closely together In the centre of the calcification the cells between the elastic fibres are still seen Note the breaks of the calcification

Tables 1 and 2 show the gross alterations in the two groups It is distinctly perceived that there exists no direct relation between the amount of adrenalin injected, and the degree of alterations Comparisons between the tables seem to indicate that the period of treatment





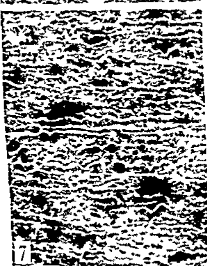


Fig 5–7

Fig 5 (van Gieson Hansen × 350) *Stage* 3 The calcium deposit is fallen out of the tissue during the preparation In the periphery the cells and fibrils are pushed away of the deposit

Fig 6 (Eskelund s modification of Go mor's old h l f h 1 10 f 4

good deal of calcium granulets in the ground substance The elastic fibres show a regular, undulated course

may be of essential importance to the changes This agrees with previous findings

In the case of the control rabbits we get a microscopical picture of aorta, in which the internal elastic membrane is distinctly seen, and the elastic fibres in media are closely packed and show a regular course In

substance is faintly metachromatic and Alcian blue positive The silver staining and PAS staining show a distinct argyrophile and PAS-positive zone or network in close relation to the elastic fibres, and

van Gieson-Hansen's staining unveils a good deal of collagen fibrils in the ground substance The cells are mostly fibroblasts, but even single muscle cells are seen No calcification were seen in the ground substance *of the control group*

After the adrenalin injections the lesions are primarily localized in the media The alterations may be classified in three stages dependent on the appearance of the calcium salt deposit

Stage 1 The medial ground substance increases sporadically but in *some places* the substance appears in pools which separate the elastic fibres In the strongly metachromatic and Alcian blue-positive pools calcium granulets are sporadically deposited among the elastic fibres, which show the usual undulated course (Fig 2) There are no degenerative of atrophic changes of the cells in the media, and no proliferation of the intima is visible The collagen fragments and reticular fibres are unchanged, but a slight increase of PAS-positive substance is often seen in close relation to the elastic fibres

Stage 2 The microscopical picture is now characterized by increased calcium deposits, sporadically distributed among the medial elastic fibres In the centre of the calcifications the elastic fibres lose their undulated course and become out-stretched, lying close to each other (Fig 4) The cells, if any, – visible in the centre of the calcareous tissue —are atrophically changed, leading to complete necrosis In the periphery of the calcium deposits, calcium granulets may be seen in the medial ground substance, and the fibroblasts and muscle cells are normal here, and no signs of atrophy or necrosis are seen (Fig 3) Around the calcium deposits it often looks as if the cells and the elastic fibrils are pushed away by the deposits, in these zones the collagen fragments are increased in number (Fig 5)

Stage 3 In this stage the microscopic picture from stage 2 is further developed The calcium deposits increase in size, and neither cells nor fibrils are seen in the centre of the calcification Breaks of the calcifications are often seen (Fig 4), or the calcium has fallen out of the tissue during the preparation, and a hole is seen in the section (Fig 6) The periphery of the deposits shows the same picture as in stage 2

Corresponding to the calcium deposits in stages 2 and 3 the intima often shows proliferation of the fibroblasts and increase of the ground substance No lipoid deposit is seen in the proliferated intima (Fig 6)

The aorta from the rabbit with 'spontaneous adrenalin changes' shows advanced gross alterations in the thoracic aorta and slight alterations in the abdominal part A big aneurysm occupies most of the anterior wall of the thoracic aorta, which is thin and unelastic The microscopic alterations correspond completely to the alterations in stages 1, 2, and 3

Comparisons between *microscopical alterations* and *the microscopical* picture show that the diffuse and sporadic accumulation of calcium granulets in the media is without any gross alterations (stage 1) Spots

in the wall correspond to small calcium deposits (stage 2), and gradually as the deposits increase in size (stage 3), the aneurysms are developed

The fully calcified adrenalin-lesions render the affected parts of the vascular wall fixed, rigid and aneurysmatic The medial tissue becomes firm in the stretched or distended position because the calcification, in so far as it destroys the media, destroys its function as an elastic recoil This point is made histologically obvious by the thinning of the wall and the straightening of the elastic fibrils in the affected areas

As mentioned, the amorphous ground substance in the control animals as well as in the treated animals reacts metachromatically, and is vigorously Alcian blue-positive, indicating a content of acid mucopolysaccharides The metachromasia is alcohol resistant The intensity of the metachromasia is considerably increased just before the calcium granulets become visible Incubation experiments with bacterial and testeshyaluronidases indicate that the acid mucopolysaccharides chiefly consist of sulphated mucopolysaccharides Not all the metachromasia is removed by testeshyaluronidases, which indicates that the sulphated mucopolysaccharides in the aortic wall are mainly chondroitin sulphates A and or C but chondroitin sulphate B and possibly heparins are present

In this experiment alterations in the peripheral arteries are not found

DISCUSSION

Josue (14) described the gross adrenalin alterations as typically atheromatous plaques with calcification and formation of aneurysms *But Klotz* (16) points out that the term "atheroma' should not be used in connection with the adrenalin lesions, as the changes are confined to the media

In the very numerous later publications on medial aortic changes, *induced partly by* adrenalin, and partly by other substances, the microscopic descriptions of the lesions are fairly identical and [illegible]

[illegible] degenerating or necrotic portions soon become *impregnated* with calcium salts, and this leads to the formation of *calcified* areas *in* the media usually localized in the inner layers of the media, *Erb* (3) *Hueper* (13), *Raab* (23)

In the present work no sign of primary necrosis has been found in the media The microscopic picture *in stage 1, and the* calcium granulets peripherally of the calcium deposits in stages 2 and 3 indicate that the deposits of *calcium* are primary That the calcareous tissue gradually may become *necrotic is a secondary* phenomenon [illegible]

ments of *Kreitmair et al* (18, 19), *Wenzel* (27), *Varela et al* (26), *Harrison* (10), *Gillman et al* (6, 7) All these authors found calcium granulets in the ground substance among the elastic fibres

A number of publications from recent years report biochemical examinations of aortic tissue from animals with "adrenalin changes" induced partly by adrenalin (*Mazzoleni et al* (21), *Lorenzen* (20)) and partly by calciferol (*Gillman et al* (6, 7), *Grant et al* (9)) All these investigations show an increase in the content of hexosamine in the treated animals, as compared to the control animals *Gillman et al* (7) *found a rise in the total aortic hexose and hexosamine to approximately* twice the control levels, and the total amount of collagen increased towards the end of the experimental period *Lorenzen* (20) found an increase in the content of hexosamine and uptake of ^{35}S sulphate, whereas the amount of hydroxyproline was unaltered or had decreased None of the authors mentioned correct their results for calcium, which no doubt explains the disagreement in the content of collagen

Many authors (1, 3, 11, 13, 23, 24, 28) compare the "adrenalin changes" in rabbits with the calcification in the media frequently found in human arteries of the muscular type (*Monckeberg* (22)) *Anitschkow* (1) asked for information of changes in the human aorta, resembling those of experimental medial alterations

It is not generally known whether a medial calcification of the human aorta with increasing age takes place, though *Klotz* (15), *Faber* (4) and *Farkas et al* (5) histologically have described such alterations in detail

In previous histochemical investigation *Bertelsen* (2) studied alterations in human aortic tissue and showed a considerable increase in the content of mucopolysaccharides, collagen, and calcium with age Calcium salts are deposited in the ground substance among the elastic fibrils, and there are no degenerative or necrotic signs in the media (Fig 7)

The diffuse alterations in human aortic tissue exhibit many points of resemblance to the local lesions in experimental "adrenalin changes" This applies to accumulation of ground substance, local increase in collagen fibrils and PAS-positive substance and the mineralization The principal difference between experimental medial changes and age-alterations in human aortic tissue is that experimental lesions tend to be localized processes, whereas in human tissue the alterations are seen through the total thoracic and abdominal aorta

The pathogenesis of the "adrenalin changes" is not clear Perhaps the damaging effect is caused by a low oxygen tension in the aortic wall due either to variations in the blood pressure or to a constriction of the vasa vasorum Possibly the increase in mucopolysaccharides is part of the process, finishing in calcification

… in human aortic tissue,
… 2)), seem to apply to the
… deposit of calcium salts
is a normal mineralization process in an organic matrix, and the calcium

crystals are deposited in the ground substance in close relation to the collagen fibrils The crystallization of the calcium salts is dependent on collagen fibrils and chondroitin sulphuric acids, as well as on the concentration of inorganic material and one or several "local factor('s), still unknown to us (*Sobel* (25), *Glimcher* (8))

As mentioned, spontaneous aortic alterations in the rabbit are similar to experimental adrenalin-induced changes Several authors have described such spontaneous changes in the medial aortic wall in rabbits, cows, and horses (4, 11, 17, 29)

SUMMARY

Adrenalin-induced changes in the aortic wall of rabbits are studied histochemically

The alterations are primarily localized in the media and are characterized by an increase in sulphated mucopolysaccharides, PAS—positive substance, collagen fibrils, and mineralization of the ground substance among the elastic fibrils

A secondary intimal proliferation is seen in relation to the medial calcification

The adrenalin induced aortic alterations are compared with the alterations in human aortic tissue with age

After completi n f th —

... associated changes on the elastic fibres at the site of crystal formation suggest that an interaction of metachromatic polysaccharides and elastin may be involved in initiating calcification

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29 *Zinse* ... orta beim Pferde ... Menschen Virch

BACTERIAL GROWTH INHIBITING EFFECT OF SOME PHLOROGLUCINOL DERIVATIVES

By

VERONICA SUNDMAN and JACOBUS SUNDMAN

Received 10 1 61

Extracts of ferns containing phloroglucinol derivatives have been long in use as anthelmintic drugs against human tape worm As it has been impossible to cultivate the tape worm *Diphyllobothrium latum in vitro,* the sensitivity of other test organisms to the therapeutic dose of anthelmintic compounds has repeatedly been tried Earthworms (*Straub* 1902, *Carlson & Bäckstrom* 1942, *Widen* 1944), small fishes (*Yagi* 1914, *Wasicky* 1924), frogs (*Straub* 1902), tadpoles (*Widen* 1944), bacteria (*Huhtala* 1949) etc have been used for the standardization of fern extracts

Huhtala (1949) established a bactericidal activity of fern extracts and of tested aspidin, flavaspidic acid, albaspidin and filixic acid The method was merely qualitative and the tested substances rather limited in number Thus we thought it worth while to test a greater number of filicinic ...

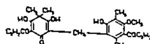
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(*Pentt* ...

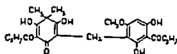
acid ... contains 6-propyl 2,3-dihydropyran 2,4 dione This latter component of the phloropyron molecule and related substances are known to have bactericidic activity and are useful as preservatives in the food industry (*Kogl & Salemink* 1952) Hence, it seemed proper to test whether this antibacterial activity of 6-propyl-2,3 dihydopyran 2,4 dione was retained in phloropyron or not

MATERIAL AND METHODS

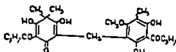
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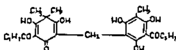
2) Desaspidin mp 152 154°



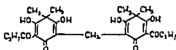
3) Paraaspidin mp 123 124°



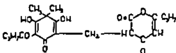
4) Flavaspidic acid mp 156°



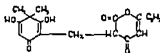
5) Albaspidin mp 148–150°



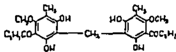
6) Phloropyron mp 111 112°



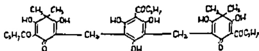
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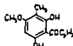
8) ψ-Aspidin mp 158 159°



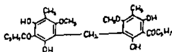
9) Filixic acid mp 180 181°



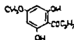
10) Aspidinol mp 142 143°



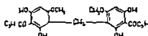
11) Methylene bis aspidinol mp 190 191°



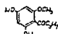
12) Desaspidinol mp 127 128°



13) Methylene bis desaspidinol mp 191 192°



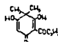
14) o Desaspidinol mp 133 134°



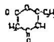
15) Filicinic acid mp 215–220° (decomp)



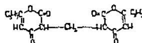
16) 3 Butyrylfilicinic acid mp 98°



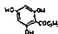
17) 6 propyl 2,3 dihydropyron 2 4 dione mp 93 94°



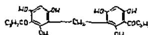
18) Methylene bis [6 propyl 2 3 dihydropyron 2 4 dione] mp 67 69°



19) Phlorobutyrophenon mp 186°



20) Methylene bis phlorobutyrophenon mp 220–225° (decomp)



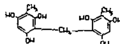
21) Phloroglucinol mp 216–218°



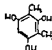
22) Methylphloroglucinol mp 213 216°



23) Methylene bis methylphloroglucinol mp ~ 230°



24) Dimethylphloroglucinol mp 162–164°



Test organisms All substances investigated were tested with *Staphylococcus aureus* ATCC 6538 Furthermore *Micrococcus flavus* ATCC 10240, *Sarcina lutea* ATCC 9341 *Brucella bronchiseptica* ATCC 4617, *Pseudomonas sp* isolated from soil and *Escherichia coli* were used in some tests

Method The substance to be tested was dissolved in aceton in the amount of 15 mg/ml 0 5 ml of this solution was added to 4 5 ml of previously sterilized buffered broth, and then aseptically diluted to give triplicate tubes with 5 ml of buffered broth respectively containing 150, 15, 1 5 0 15 μg/ml of the substance It was found necessary to use a buffer concentration high enough to prevent every change of pH during growth since the solubility of many of the substances tested decreases with decreasing pH, thus possibly giving a turbidity read as growth in the method Sorensen phosphate buffer in a final concentration of 1/15 mole per liter was found sufficient When not otherwise stated the tests were performed at pH 7 8 Then two control tubes without added phloroglucinol derivative were set up in triplicate Further, control tubes with 1 per cent acetone were necessary, since according to the dilution scheme the tubes with 150 μg/ml of the substance to be tested also contained 1 per cent acetone This acetone amount was found to increase the turbidity of the tubes during gorwth[1] When growth was noticed in the tubes with 150 μg/ml these control tubes were used for the estimation of maximal growth Cf below under evaluation of results

The tubes were inoculated with 1 drop of a 20 hour broth culture of the test organism and incubated at 37° (when *Pseudomonas sp* was used as test organism the tubes were incubated at 28°) The turbidity of the tubes was measured after 24 hours with an EEL nefelometer calibrated to 100 against a bariumsulphate suspension standard

Evaluation of results The mean values of the triplicate turbidity readings reduced by the reading for sterile broth were calculated as per cent of maximal growth[2] and plotted on semilogaritmic paper against the concentration of the tested substance laid off on the logaritmic abscissa From thus obtained curves the activity of the substance termed "D_{50}" is estimated as the concentration causing a 50 per cent depression of growth

RESULTS AND DISCUSSION

In Figures 1 6 are shown the growth-concentration-curves for the substances tested and in Table 1 are listed the figures for growth depression activity (D_{50}) in decreasing order as estimated from those curves Among the substances tested aspidin was found to be the phloroglucinol derivative most active against *S aureus*, cf Fig 1 This is in conformity with earlier work on the biological activity of the fern extractives, cf *Huhtala* 1949 p 207, *Widen* 1944 p 64 The latter author gives the following list of phloroglucinol derivatives effective against earthworms, in order of decreasing toxicity at pH 8 aspidin, flavaspidic

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[1] Acetone concentrations of 0 1 per cent and less had no influence on the turbidity of the tubes

[2] Maximal growth = turbidity of control tubes reduced by the reading for sterile substrate

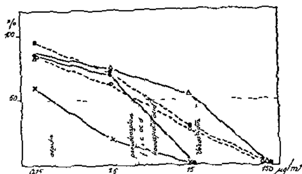


Fig 1

Growth of *S aureus* at 37° in buffered broth with increasing amounts of aspidin, paraaspidin (pseudoaspidin), filixic acid, flavaspidic acid, and desaspidin, respectively

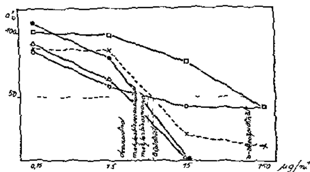


Fig 2

Growth of *S aureus* at 37° in buffered broth with increasing amounts of desaspidinol, methylene-bis desaspidinol, methylene bis aspidinol, aspidinol, and ortho desaspidinol, respectively

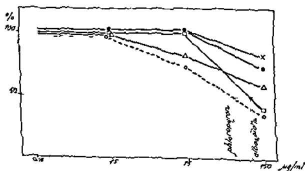


Fig 3

●—● = methylene bis-{6 propyl 2,3 dihydropyran 2,4 dione}

×—× = 6 propyl 2 3-dihydropyran 2 4 dione

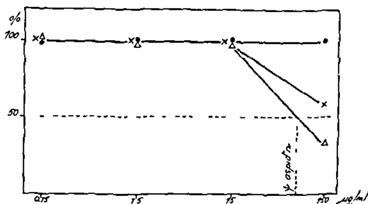


Fig 4
Growth of *S aureus* at 37° in buffered broth with increasing amounts of ψ-aspidin butyrylfilicinic acid, and filicinic acid, respectively
×—× = butyrylfilicinic acid
●—● = filicinic acid

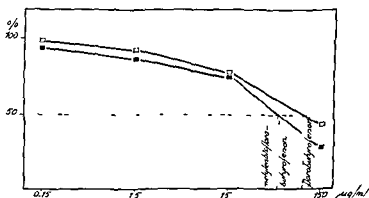


Fig 5
Growth of S *aureus* at 37° in buffered broth with increasing amounts of *methylene-*bis-phlorobutyrophenon, and phlorobutyrophenon respectively

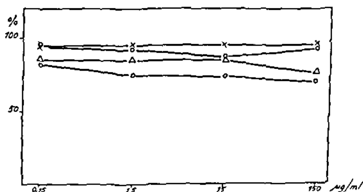


Fig 6
Growth of S *aureus* at 37° in buffered broth with increasing amounts of methylene-bis methylphloroglucinol phloroglucinol methylphloroglucinol and dimethylphloroglucinol, respectively
○—○ = methylene-bis methylphloroglucinol
△—△ = phloroglucinol
●—● = methylphloroglucinol
×—× = dimethylphloroglucinol

acid, filixic acid, aspidinol albaspidin As seen from Table 1 this corresponds to our results at pH 7 8 with the exception that we found filixic acid slightly more active than flavaspidic acid

Substances which contain a 3 butyrylfilicinic acid component bound by a methylene bridge to a derivative of phlorobutyrophenon *viz* aspidin, paraaspidin desaspidin and flavaspidic acid are found among the nine most active substances listed in Table 1 Though filixic acid is a trimeric compound it may be included in this group because it is composed of one flavaspidic acid unit and one 3 butyrylfilicinic acid unit Since the latter is only slightly effective against *S aureus* $D_{50} = > 150$ μg ml, the flavaspidic acid part of the molecule is obviously responsible for the activity of filixic acid The molecular structures of the remaining four substances at the top of the Table 1, *i e*, desaspidinol, methylene bis-desaspidinol aspidinol, and methylene bis aspidinol have, as a common feature, the methoxyl group in para position to the butyryl group

TABLE 1

Growth Inhibiting Activity of Substances Tested against S aureus at pH 7 8
D_{50} In heating Concentration in μg ml for Causing a 50 per cent Depression of Growth

Aspidin	0.3
Desaspidin 1	2 6
Paraaspidin	3 0
Filixic acid	3 4
Me bis desaspidinol	3 6
[illegible]	4 8
	4 9
	5 4
	18
	54
	62
ψ Aspidin	76
ortho Desaspidinol	90
Albaspidin	100
[illegible]	100
	>150 *
	>150
	>>150 †
	>>150
[illegible] acid	§
Phloroglucinol	—
Methylphloroglucinol	—
Dimethylphloroglucinol	—
Me bis methylphloroglucinol	—

* Growth at 150 μg/ml less than 60 per cent
† Growth at 150 μg/ml greater than 70 per cent
§ No effect noticed at 150 μg/ml

The [illegible]

[illegible] investigated by *Kogl & Salemink*

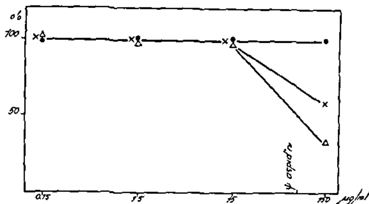


Fig 4

Growth of *S aureus* at 37° in buffered broth with increasing amounts of ψ aspidin butyrylfilicinic acid and filicinic acid respectively

X—X = butyrylfilicinic acid

●—● = filicinic acid

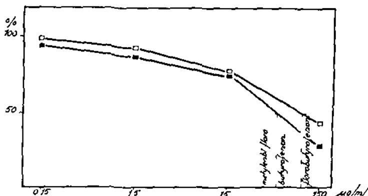


Fig 5

Growth of *S aureus* at 37° in buffered broth with increasing amounts of methylene bis phlorobutyrophenon and phlorobutyrophenon respectively

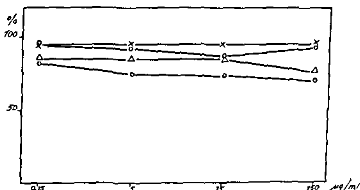


Fig 6

Growth of *S aureus* at 37° in buffered broth with increasing amounts of methylene bis methylphloroglucinol phloroglucinol methylphloroglucinol and dimethylphloroglucinol respectively

○—○ = methylene bis methylphloroglucinol

△—△ = phloroglucinol

●—● = methylphloroglucinol

X—X = dimethylphloroglucinol

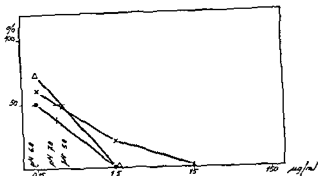


Fig 7
Growth of *S aureus* at 37° in buffered broth of varying pH with increasing amounts of aspidin

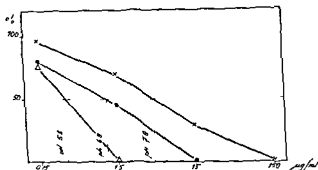


Fig 8
Growth of S *aureus* at 37° in buffered broth of varying pH with increasing amounts of flavaspidic acid

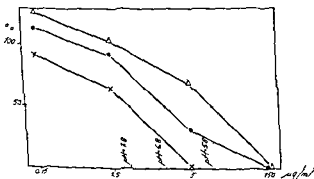


Fig 9
Growth of S *aureus* at 37° in buffered broth of varying pH with increasing amounts of desaspidinol

For 6-propyl-2,3-dihydropyran-2,4-dione was found a D_{50} value of >150 µg/ml From the data reported by *Kogl & Salemink* (1952) a corresponding value of some 30 µg/ml can be evaluated The results, however, are not directly comparable, we used a different substrate and worked at pH 7 8, they at pH 7

The butyryl unit has been found to contribute to the bactericidal activity of the phloroglucinol derivatives When the butyryl unit is removed from phloropyron, desbutyrophloropyron is obtained As seen in Fig 3 the latter substance is less poisonous than phloropyron A similar effect of the butyryl unit can be found by comparing the curves for 3-butyryl-filicinic acid and filicinic acid in Fig 4 and the curves for phlorobutyrophenon and phloroglucinol in Figs 5 and 6, respectively

TABLE 2

Growth Inhibiting Activity of Substances Tested at Different pH Levels against S aureus D_{50} Indicating Concentration in µg/ml for Causing a 50 per cent Depression of Growth

Substance	D_{50}		
	pH 7 8	pH 6 8	pH 5 8
Aspidin	0 3	0 2	0 3
Paraaspidin	3 0	1 7	2 0
Desaspidin	19	0 7	0 4
Flavaspidic acid	4 5	1 1	0 3
Desaspidinol	2 5	7 2	28

It is known from earlier experiments with earthworms (*Widén* 1944) that the poisonous properties of some phloroglucinol derivatives are dependent on the prevailing pH We investigated whether or not any effect of pH on the bacterial growth inhibition of the phloroglucinol derivatives could be established The results are given in Table 2 where D_{50}-figures for five substances at different pH levels are listed As seen *from the table, the action of aspidin and of paraaspidin is not* much changed when pH varies between 5 8 and 7 8 The growth inhibiting activity of desaspidinol is increased with increasing pH For flavaspidic acid and desaspidinol the opposite is true, the bactericidal effect being higher at lower pH These three different types of pH dependence are illustrated in Fig 7–9 where the growth-concentration curves at different pH levels for aspidin, flavaspidic acid and desaspidinol are presented As seen in Fig 9, desaspidinol in low concentration at pH 5 8 and 6 8 stimulates the growth of *S aureus* This repeatedly observed effect has not been noticed for any other of the substances tested

The effect of pH on the poisonous nature of the phloroglucinol derivatives has *been explained (Widén 1944) by* assuming that the undissociated molecule is more poisonous than the corresponding ion Thus, a weakly acid substance would show its maximal poisonous

be pointed out that during the course of this investigation, large clinical activity of a substance from the results found with bacteria it should experiments with many thousands of patients were performed with pure desaspidin as anthelminticum In part the impulse behind these experiments was the strong bactericidal effect found for desaspidin in slightly acid environment The clinical experiments the results of which will be published elsewhere have been much more successful than any previous experiments with mixtures or pure substances of fern origin So there seems to be criteria for indicating that the bacterial test with *S aureus* could be a useful tool in the search for anthelmintic drugs against *Diphyllobothrium latum*

SUMMARY

The bacterial growth inhibiting activity of 24 phloroglucinol derivatives was investigated nefelometrically with *Staphylococcus aureus* as test organism The most active substance was found to be aspidin which caused a 50 per cent growth inhibition at a concentration of 0 15 μg ml The growth inhibition is dependent on pH No bactericidal effect against gram negative bacteria was noticed at the concentrations used The relationship between the growth inhibiting effect and the constitution of the pholoroglucinol derivatives is discussed

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2 *Huht* [illegible]

3 *Kögl* [illegible]

4 *Penti* [illegible] 1932

5 [illegible]

6 [illegible] gische Studien uber die Substanzen der Fil[illegible] Arch exp Path [illegible]

7 [illegible]

8 [illegible]

9 *Jagi S* Physiologische Werthestimmung von Filixsubstanzen und Filixextrakten Z ges exp Med 3 64 1914

action at a higher pH than a more acid compound This was found to be true for the phloroglucinol derivatives tested at different pH levels The acidity of the compounds may be characterized by the relationship between Rf value and pH of the paper, found in a chromatographic study of the phloroglucinol derivatives in *Dryopteris* ferns (*Penttila & Sundman* 1960 a) According to this study, the substances tested can be ordered after decreasing acidity as follows flavaspidic acid, desaspidin, aspidin and desaspidinol

It should be noticed that when the substances with aspidin-like structure *viz* aspidin, paraaspidin, desaspidin and flavaspidic acid are allowed to act on *S aureus* at the pH most favorable for growth inhibition respectively, they exhibit a more uniform activity (D_{50} varying between 0 2 and 1 7 µg/ml) than that indicated by Table 1 where D_{50} as determined at pH 7 8 varies between 0 3 and 18 µg/ml

In order to confirm the observation of *Huhtala* (1949) that *Escherichia coli* is much less sensitive to the phloroglucinol derivatives of fern origin than is *S aureus* we compared the sensitivity of these two organisms to seven of the substances found most active In no case could any growth inhibiting effect against *E coli* be noticed These experiments were performed with 150 µg/ml as maximal concentration in conformity with the method used throughout this investigation

TABLE 3

Growth Inhibition of Aspidin Tested at pH 7 8 against Different Bacteria D_{50} Indicating Concentration in µg/ml for Causing a 50 per cent Depression of Growth

Test	D_{50}
Gram positive	
Staphylococcus aureus	0 2
Sarcina lutea	0 4
Micrococcus flavus	4 6
Gram negative	
Escherichia coli	–*
Brucella bronchiseptica	
Pseudomonas sp	–

* No action could be noticed at 150 µg/ml

The difference in sensitivity established for *E coli* and *S aureus* was found also between other gram-negative and gram-positive bacteria In Table 3 is seen the result of a comparison of the sensitivity to aspidin between 3 gram-positive and 3 gram-negative bacteria respectively As seen in the table, no effect of aspidin on the growth of the gram-negatives could be established, whereas the gram positives were all inhibited in small concentration These results are in conformity with the general rule that phenolic compounds are more poisonous to the gram-positive than to the gram-negative bacteria

As to the possibility of drawing conclusions about the anthelmintic

be pointed out that during the course of this investigation, large clinical activity of a substance from the results found with bacteria it should experiments with many thousands of patients were performed with pure desaspidin as anthelminticum In part the impulse behind these experiments was the strong bactericidal effect found for desaspidin in slightly acid environment The clinical experiments the results of which will be published elsewhere have been much more successful than any previous experiments with mixtures or pure substances of fern origin So there seems to be criteria for indicating that the bacterial test with *S aureus* could be a useful tool in the search for anthelmintic drugs against *Diphyllobothrium latum*

SUMMARY

The bacterial growth inhibiting activity of 24 phloroglucinol derivatives was investigated nefelometrically with *Staphylococcus aureus* as test organism The most active substance was found to be aspidin which caused a 50 per cent growth inhibition at a concentration of 0 15 μg ml The growth inhibition is dependent on pH No bactericidal effect against gram negative bacteria was noticed at the concentrations used The relationship between the growth inhibiting effect and the constitution of the pholoroglucinol derivatives is discussed

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FROM THE BACTERIOLOGICAL DEPARTMENT OF SERAFIMERLASARETTET STOCKHOLM (HEAD ARNE LITHANDER M.D.)

ANTITOXIC IMMUNITY AS PROTECTION AGAINST STAPHYLOCOCCIC INFECTION

An Experimental Study in Mice and Rabbits

By

ARNE LITHANDER

Received 21 iii 61

Only very few investigators consider the serum alpha antitoxin formed at immunization with staphylococcic toxoid or toxin to be of value in the defense against staphylococcic infections Their conclusion is based on the results of animal experiments in which animals immunized with staphylococcic toxoid or toxin survived challenge injections of the staphylococci in the same doses that were lethal in non immunized controls One counter-argument that has been presented is that the staphylococcic toxin in the staphylococcic suspensions used for the challenge injection was the real cause of death of the controls in the experiments in question Furthermore, the reason the immunized animals survived longer than the controls is said to be that the toxin of the staphylococci in the challenge injection was neutralized by the antitoxin However, the same animals later succumbed to infection Thus, *Ellek* (1) claimed that immunization with staphylococcic toxin does not protect against infection with staphylococci but only makes the infection more protracted without preventing a fatal outcome In animal experiments, however, he generally found a high level of staphylococcic alpha antitoxin to be effective against intoxication with staphylococcic toxin

Fisher (2) immunized mice with staphylococcic toxin and formalized toxin After having infected the mice intraperitoneally with coagulase producing staphylococci, he found that animals immunized with a large amount of toxin were protected against death by infection In these animals the level of alpha antitoxin in serum was higher than in the other animals Fisher's observation time of seven days, however, appears to be too short to decide whether the animals in his experiments would have survived indefinitely

In children and adults with pyoderma, *Dobias et al* (3) found the resistance to be related to a high staphylococcic alpha antitoxin content in serum They emphasized that a high antitoxin content is not

he only important factor, but that it may serve as an index of resistance to infections Some workers have shown that a high content of taphylococcic alpha antitoxin in persons suffering from boils prevents ecurrence of the condition

An attempt has been made in the present investigation to determine whether a high level of alpha antitoxin in serum of animals immunized with staphylococcic toxoid is of value in preventing infection with virulent staphylococci

MATERIALS AND METHODS

The experiments were made both on rabbits weighing approximately 2 5 kg and on white mice weighing 20 gm The rabbits were divided into four series In each series one group of the rabbits was untreated and served as controls one or two other groups of rabbits were immunized by subcutaneous injections of staphylococcic toxoid containing 10 I U per ml or dilutions of the toxoid The injections

eek after the last injection half of the animals in each group of immunized mice and of the controls were killed The antitoxin level was then

of a 5 per cent solution of gastric mucin The staphylococci used for the challenge of all the mice and of the rabbits in Series 1 and 2 were untreated The staphylococci for Series 3 and 4 of the rabbits were washed in saline four times before the inject the staphyloco

RESULTS

Tables 1 and 2 reveal that the rise in the antitoxin level in serum was greater in mice and rabbits immunized with undiluted toxoid than in those immunized with diluted toxoid In addition, the dilution 1/10 gave a better result in mice than the dilution 1/100 The antitoxin level was very low in the controls

TABLE 1

Mice Challenged by the Intraperitoneal Route with 300 Million Untreated Staphylococcus aureus of a Coagulase Producing Strain

Immunizing agent	Dilution	Serum alpha antitoxin titre before challenge	Challenge dose Number of untreated staphylococci Millions	Number of mice	Number of survivors after			Per cent survivors after		
					1 day	2 days	14 days	1 day	2 days	14 days
Toxoid	1/1	2 25 3 0	300	24	24	22	22	100 0	91 5	91 5
	1/10	1 3 2 25	300	24	23	17	14	95 8	70 8	58 3
	1/100	0 65 1 3	300	24	19	16	15	79 2	66 7	62 5
Nil (control)		< 0 1	300	30	10	8	7	33 3	26 7	23 2

It is clear from Table 1 that there was a significant protection against the staphylococci introduced by injection in all groups of vaccinated mice. Many of the controls died the day after the challenge injection, a few more succumbed during the subsequent 13 days. All of the mice in the group vaccinated with undiluted toxoid were still alive one day after the injection, and only two of them died in the first two weeks after vaccination. In the other two groups of vaccinated mice, the protection was less, but they still survived significantly longer than the controls. The relation between the antitoxin level in blood and the survival of the mice appears to be rather close.

The results of the experiments with rabbits (Table 2) are less significant due to the smallness of the material. Nevertheless the immunized rabbits in all the series, withstood the challenge much better than the controls. It would thus appear that the rabbits immunized by toxoid or dilutions thereof were better protected against the challenge both with washed and untreated staphylococci. As in the experiments with mice, only very few of the immunized rabbits died during the first few days after the challenge injection. Among the controls there seems to be a difference between Series 1 and 2 in which the rabbits were challenged with a toxin containing suspension of untreated staphylococci and Series 3 and 4 in which toxin free, washed staphylococci were used as challenge. In general the former succumbed earlier to the challenge than the latter.

The following results were obtained by culturing repeated samples of blood from the rabbits. The blood of the controls and of the immunized rabbits usually contained a great number of staphylococci in the first few days after the challenge. At death also, the controls as a rule had a very heavy contamination of staphylococci in the blood. There was also a rather heavy contamination of staphylococci in the blood at death in the immunized rabbits which died before the end of the first week, but this was less pronounced than in the corresponding controls. Immunized rabbits which died after more than one week showed very few staphylococci in the blood at death. All rabbits which survived throughout the observation time—seven weeks—showed very few or no staphylococci at all in the blood at the end of this period. In the immunized rabbits which survived, the number of staphylococci was markedly reduced as early as one week or even less after the challenge.

Autopsy as a rule revealed marked differences between the adrenals of immunized rabbits and controls. The adrenals of the controls in Series 1 and 2 which succumbed to the challenge showed great changes. They were superficially red or reddish and showed hemorrhages in section. In the controls in Series 3 and 4 the adrenals showed only slight changes or no changes at all. There were generally no changes in the adrenals of immunized rabbits which succumbed after challenge. Two of them showed very slight adrenal changes.

TABLE 1

Mice Challenged by the Intraperitoneal Route with 300 Million Untreated Staphylococcus aureus of a Coagulase Producing Strain

Immunizing agent	Dilution	Serum alpha antitoxin titre before challenge	Challenge dose Number of untreated staphylococci Millions	Number of mice	Number of survivors after			Per cent survivors after		
					1 day	2 days	14 days	1 day	2 days	14 days
Toxoid	1/1	2 25 3 0	300	24	24	22	22	100 0	91 5	91 5
	1/10	1 3 2 25	300	24	23	17	14	95 8	70 8	58 3
	1/100	0 65 1 3	300	24	19	16	15	79 2	66 7	62 5
Nil (control)		< 0 1	300	30	10	8	7	33 3	26 7	23 2

DISCUSSION

As a rule, immunization with staphylococcic toxin or toxoid is reported to provide resistance to staphylococcic toxin, but not to infections with staphylococci It is generally agreed that in infections a rise in the alpha antitoxin in the blood neutralizes the toxin It does not prevent the infection from leading to death, however, but only slows down its progress by protecting the organism against the toxin

The writer's experiments with both mice and rabbits revealed a distinct difference between immunized animals, on the one hand, and non-immunized controls, on the other Many more immunized animals than controls survived the entire period of observation This suggests that the immunization had a protective effect Deaths of immunized animals following challenge injections were spread over the whole observation period This was true both when washed and untreated staphylococci were used for the challenge On the other hand, the controls which were given untreated staphylococci usually died shortly after the chal lenge injection In the experiments with rabbits, there was also some spread of deaths of controls injected with washed staphylococci

One explanation of these observations is that the preformed toxin in the suspension of untreated staphylococci quickly killed most animals with a low content of antitoxin In immunized animals, on the other hand the antitoxin neutralized the preformed toxin and thus protected these animals against the rapidly lethal effect of the toxin In im munized animals surviving the whole observation time the high antitoxin concentrations also protected them against the toxin formed *in vivo* by the staphylococci injected into them With this protection, the animals could form antibacterial antibodies which later collaborated with the nonspecific defence forces of the organism to overcome the infection The length of the observation periods—seven weeks for the rabbits and two weeks for the mice—should have been sufficient to determine whether the infection was definitively extinguished

The fact that occasional controls survived indicates that factors other than an increased alpha antitoxin level are of significance, even though it appears from the results that the antitoxin content is of major im portance The number of rabbits is too small to permit an evaluation of the differences in the antitoxin content of the immunized rabbits, but even as low a level as less than five units per milliliter is probably significant

Further evidence that an increased antitoxin content protects the organism against staphylococcic infection is provided by the results of the blood cultures made repeatedly in each rabbit In the majority of the controls, there was still a great number of staphylococci in the blood at death The staphylococcic count had decreased, but was still rela tively high in immunized rabbits which succumbed within one week of the challenge injection In the surviving rabbits all or practically all

TABI F 2

Rabbits Challenged by Intravenous Injection with Staphylococcus aureus of a Coagulase Producing Strain In Series 1 and 2 Untreated in Series 3 and 4 Washed Staphylococci

Series no	Toxid dilution	Serum alpha anti toxin titre before challenge		Number of injected staphylo cocci Millions	Num ber of rabbits	Number of survivors after									
						days							weeks		
		Mean	Range			1	1	3	4	5	6	7	2	3	7
				Untreated											
1	1/1	12 8	10 16	7,500	4	4		–	–	4	3	–	–	–	3
	1/10	7 4	7– 8	7,500	5	5	4	–	4	3	–	–	–	–	3
	Nil (control)	<0 1	<0 1	7 500	5	3	1		–	–	–	–	–	–	1
2	1/10	4 6	4– 5	6 000	5	5		–	–	–	–	–	–	–	5
	Nil (control)	<0 1	<0 1	6 000	5	3	0	–	–	–	–	–	–	–	0
				Washed											
3	1/1	9 0	5 25	8 000	9	9	8	8	7	7	5	4	2	–	2
	Nil (control)	<0 1	<0 1	8 000	10	6	4	2	–	2	1	0	–	–	0
4	1/1	12 8	6 5 20	6 000	8	8			–	–	–	8	7	–	7
	1/10	4 0	2 2 5	6 000	8	8	–		–	–	–	8	7	6	6
	Nil (control)	<0 1	<0 1	6 000	8	5	4	–	–	–	4	3	3	1	1

COMPLEMENT FIXATION WITH FORMALIN TREATED POLIOVIRUS ANTIGENS

By

JOHN PAPAPANAGIOTOU[1] and HERDIS VON MAGNUS

Received 25 ii 61

For the routine assay of the potency of formalin treated polio vaccines *in vivo* methods are ordinarily used Such methods are both time consuming and costly, and an exploration of the possibility of using *in vitro* methods for the assay of the potency of the polio vaccine has therefore been desirable

Several *in vitro* tests have been described *Krech* (1, 2) developed a test for the measuring of the antigenic potency in terms of the antibody combining power of inactive virus Antibody combining tests have been used also by others (3–5)

Hare & Warren (6) and *Warren et al* (7) have suggested the application of the complement fixation (CF) technique not only as a supplementary assay of potency of the final vaccine but also as a means of following the antigenicity during the various stages of processing These authors have investigated the possible use of the CF technique in two ways, firstly by the determination of the ability of a vaccine to elicit CF antibodies in various animal species and secondly, by the direct measurement of viral CF antigens present in the vaccine

In the present study an attempt was made to evaluate the latter of the two above mentioned applications of the CF technique

MATERIALS AND METHODS

Tissue Culture

The preparation of trypsinized monkey kidney tissue cultures has been described in detail previously (8)

Poliovirus Strains

The ... Type III) strains were us[ed] ... [str]ains employed in this lab[oratory] ...

Preparation of CF An[tigens]

For the prepara[tion] ...
Roux bottles were ...

[1] Present address National University of Athens Faculty of Medicine Department of Microbiology Athens Greece

traces of staphylococci in the blood had disappeared one to two weeks after the challenge injection. This course of events may have been due to the fact that the immunized rabbits—particularly those which survived—were able to eliminate the staphylococci injected into them and that most of the controls lacked this ability.

Evidence that intoxication caused the sudden deaths is provided by the fact that the adrenal lesions were more extensive in controls than in immunized rabbits, and in controls injected with untreated staphylococci, *i.e.* mixed with pre-formed toxin, than in controls injected with washed staphylococci. The circumstance that the lesions were less extensive in animals which succumbed later after the challenge injection suggests that the intoxication was of less importance in these animals.

SUMMARY

(1) Rabbits and mice immunized with staphylococcic toxoid as well as untreated controls, were injected with coagulase-positive staphylococci, which in some series were freed from pre-formed toxin by washing and in others were untreated.

(2) The immunized animals survived for a much longer period than the untreated controls.

(3) The controls in the rabbit experiments given challenge injections with washed staphylococci as a rule lived longer than those injected with untreated staphylococci.

(4) Early deaths in the controls were due to the effect of the toxin. The elevated alpha antitoxin in the blood of the immunized animals gave protection against the pre-formed toxins immediately after the challenge injection. Thereafter, the increased antitoxin was a protective force against toxin formed later, permitting the organism to develop greater antibacterial resistance.

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THE ENTEROVIRUS DEPARTMENT, STATENS SERUMINSTITUT COPENHAGEN

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In the present study an attempt was made to evaluate the latter of the two above mentioned applications of the CF technique

MATERIALS AND METHODS

Tissue Culture

The preparation of trypsinized monkey kidney tissue cultures has been described in detail previously (8)

Poliovirus Strains

The Brunhilde (Type I) MEF 1 (Type II) and Saukett (Type III) strains were used for the preparations of CF antigens These are the strains employed in this laboratory for the production of Salk type polio vaccine (8)

Preparation of CF Antigens from Live Poliovirus

For the preparation of antigens the following method has been adopted (9) Roux bottles were seeded with approximately 10^7 trypsinized monkey kidney cells

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(MK cells) in about 100 ml of medium 199 containing 2 per cent horse serum as well as antibiotics. After 4 to 5 days of incubation at 36° C the medium was replaced by fresh outgrowth medium of the following composition (9):

Hanks' solution	ca	90.0 per cent
Lactalbumin hydrolysate		0.5 – –
Glucose		0.45 – –
Yeast extract		0.1 – –
Sodium bicarbonate		0.11 – –
Calf serum		10.0 – –

After a total incubation of 8–9 days the outgrowth medium was removed and the cell sheet was washed with Hanks' balanced salt solution. After washing 50 ml of maintenance medium were added consisting of bovine amniotic fluid with 1.2 per cent calf serum, and the bottles were inoculated with 1 ml of undiluted tissue culture seed virus. The inoculated bottles were incubated at 36° C for 48 hours at which time complete degeneration of the cells had occurred. The infected fluids were harvested and pooled by virus type. The cell debris was removed by horizontal centrifugation at 2500 r.p.m./30 min. in a refrigerated centrifuge, and the supernatant was treated with fluorocarbon (Freon 113) (9). The Freon treated antigen was titrated against the homologous monkey antiserum and stored at —20° C. At this temperature the CF antigen was found to retain its potency for at least 6 months.

Formalin Treated Poliovirus

To infectious tissue culture fluid cleared by centrifugation at 2500 r.p.m./30 min formalin was added in a final concentration of 1:4000. The mixture was kept at 37° C for varying periods of time. Before use in CF tests the formalin treated tissue culture fluids were dialyzed against Hanks' balanced salt solution at 4° C.

Concentration of Formalin Treated Polio Vaccine in the Ultracentrifuge

Concentration of polio vaccines was performed in a 'Spinco' (Model L) ultracentrifuge. The fluids were placed in lusteroid tubes with aluminium caps in amounts of 30 ml per tube and centrifugation was carried out at 78000 × G (30000 r.p.m.) for 180–240 minutes in the cold in a rotor No. 30. Gelatin in a concentration of 0.06 per cent was added to the fluid before centrifugation according to the method of *Baron* (9, 10). After centrifugation the gel-like pellet was resuspended in warm (37° C) Hanks' solution to give the desired concentration, usually 30 × or 10 ×. *The faintly opalescent concentrates* were then clarified by 5 minutes of centrifugation at 2000 r.p.m. We have found that placing the tube in a 37° C water bath for 5 minutes before centrifugation helped to dissolve the aggregates of gelatin (9).

Hyperimmune Monkey Sera

The preparation of monkey hyperimmune sera has been described previously (11). The same lots of monkey sera were used throughout this study. These sera were kindly made available by Dr. *Annelise Godtfredsen* of this institute.

Before use in the CF test the sera were diluted 1:8 in veronal buffered saline and inactivated at 60° C for 30 minutes. The complement fixing (CF) antibody titer of the sera against the homologous CF live virus antigens were 1:1024–1:2048 for type I, 1:256–1:512 for type II and 1:512–1:1024 for type III.

Technique for CF test has been described in detail in an earlier communication (9). Veronal buffered saline (12) was used as diluent.

The live virus CF antigens were titrated in block titrations with monkey antisera. For the poliovirus type I and II 2–4 units of antigen were used, and for the type III 4 units of antigen (9).

The CF tests were performed in glass tubes (Widal tubes) with a total volume of 0.5 ml per tube. An amount of 0.1 ml each of antigen, serum dilution and complement was allowed to react overnight at +4° C. Then 0.2 ml of sensitized 1 per cent

sheep erythrocytes was added to each tube followed by 30 minutes of incubation at 37° C. All necessary controls were included The test was read after overnight standing at +4° C. Fixation greater than 2 + (less than 50 per cent hemolysis) was considered as positive reaction

EXPERIMENTAL

Polio Vaccine Used as Antigen in CF Test

Fifteen pools of monovalent polio vaccines (five of each type) were examined in CF tests with monkey hyperimmune sera The vaccines studied represent the product routinely prepared in this laboratory for use in humans

TABLE 1
Formalin Inactivated Poliovirus (Vaccine) as Antigen in CF Tests with Potent Monkey Antisera

Vaccines	No	Dilution of vaccine	Type of monkey antiserum		
			Type I	Type II	Type III
Type I (Brunhilde)	524	1 1	1024*	< 16	< 16
		1 2	32		
	527	1 1	1024	< 16	< 16
		1 2	128		
	540	1 1	512	< 16	< 16
		1 2	< 32		
	547	1 1	1024	< 16	< 16
		1 2	128		
	548	1 1	1024	< 16	< 16
		1 2	128		
Type II (MEF 1)	509	1 1	< 16	512	< 16
		1 2		64	
	511	1 1	< 16	256	< 16
		1 2		32	
	513	1 1	< 16	256	< 16
		1 2		< 32	
	550	1 1	< 16	128	< 16
		1 2		< 32	
	551	1 1	< 16	128	< 16
		1 2		32	
Type III (Saukett)	519	1 1	< 16	< 16	256
		1 2			< 32
	521	1 1	< 16	< 16	256
		1 2			< 32
	533	1 1	< 16	< 16	256
		1 2			< 32
	535	1 1	< 16	< 16	256
		1 2			< 32
	537	1 1	< 16	< 16	256
		1 2			< 32
Live virus type I			1024 2048		
type II				256	
type III					512 1024

Titers expressed as reciprocals of serum dilution

Briefly, the technique of the processing of the vaccine is as follows Crude, infected TC fluid is Seitz-filtered and inactivated for 12 days with formalin 1 4000 at 37° C and pH 7 A second Seitz-filtration is performed on day 6 of the inactivation At the end of the inactivation period formalin is neutralized by the addition of bisulphite The 3 types of poliovirus are represented in the final trivalent pools as follows Type I 3 parts, type II 1 part, and type III 2 parts

The fifteen monovalent samples were examined without concentration and dialyzation in the CF test (Table 1) It was found that undiluted vaccine gave a considerable degree of type specific fixation with potent homologous antisera The same batches of vaccine were also examined after dialyzation and 14 were found to give the same serum titer Only one vaccine (No 509, type II) gave a 4-fold lower serum titer This particular batch of vaccine was before dialyzation slightly anti complementary (anti-C') Since any trace of anti-C' activity was found to be removed from the vaccines by dialyzation, the vaccines were subsequently always dialyzed before testing

TABLE 2
Monovalent Type II Vaccines as Antigen in CF Tests with Monovalent Type II Guinea Pig Sera

Antigen		CF titer of 7 monovalent type II guinea pig sera							CF titer of monovalent monkey antisera		
No	Type	4771	4772	4773	4775	4796	4798	4799	I	II	III
Vaccine 549 L	II	32*	8	16	16	8	<2	2	–	64	–
Vaccine 550 L	II	32	16	16	16	8	<2	2		64	–
Vaccine 567 L	I	<2	<2	<2	<2	<2	<2	<2	1024	–	–
Vaccine 555 L	III	<2	<2	<2	<2	<2	<2	<2			256
Live CF antig 182	II	128	128	64	64	32	2	8	–	256	

* Titers as reciprocals of serum dilution
– Not done

In contrast, *Warren et al* (7) have previously found that the amount of antigenic material in commercial vaccines is insufficient to permit their use in practical CF procedures without concentration For their studies they used guinea pig antisera To examine whether the positive results obtained in the present experiment with *unconcentrated* monovalent vaccines were due to the use of potent hyperimmune monkey sera in our CF test, a number of type II guinea pig sera were examined in parallel against two type II vaccines, a live type II Freon treated CF antigen, a type I vaccine, and a type III vaccine Monkey hyperimmune sera of all 3 types were also included in the experiment The results are shown in Table 2 It will be seen that it is possible to measure CF antibodies in the guinea pig sera employing type II monovalent polio vaccines as antigens However, the serum titers were 4 times higher with live type II virus antigen, indicating that less than 1 unit of antigen

was present in the vaccine The negative results obtained with type I and type III antigens which were included as controls show that the reaction is type specific

The results obtained with *various* vaccine antigens were usually easily reproducible in repeated tests However, in some instances the CF serum titers were found to vary distinctly from one experiment to another This difficulty was first encountered when 23 type *III* mono valent polio vaccines were employed as antigens in a CF test In the first experiment is was found that while 18 of the vaccines represented good CF antigens giving a titer of 1 128–1 256 with the homologous monkey antiserum one vaccine gave a somewhat lower titer (1 64) and 4 vac cines did not react at all When *the tests with these 4 antigens (No* 556, 557, 560 and 561) were repeated the results were found to vary a great deal It will thus be seen from Table 3 that while in two tests (Exp 1 and 4) the titers were < 32, the serum titers in Exp 3 ranged from 64 to 256

A scrutinizing of the experimental data indicated that the variation in serum titer might possibly be correlated with small differences in the amount of complement employed in the titration of low potency CF antigens, and an experiment was therefore set up to study the effect of varying amounts of complement

TABLE 3

Repeated CF Tests with Type III Vaccines as Antigen

Exp no	*CF titer * of type III monkey serum*			
	Vaccine number			
	556	557	560	561
1	< 32	< 32	< 32	< 32
2		< 32	< 32	64
3	64	128	64	256
4	< 32	< 32	< 32	< 32

* Titer as reciprocals of serum dilution
Not done

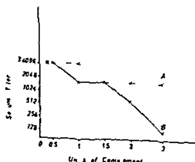


Fig 1

CF test with two different polio I antigens (A and B) in the presence of varying amounts of complement a anticomplementary

Briefly, the technique of the processing of the vaccine is as follows Crude, infected TC fluid is Seitz-filtered and inactivated for 12 days with *formalin 1 4000 at 37° C and pH 7 A second Seitz-filtration* is performed on day 6 of the inactivation At the end of the inactivation period formalin is neutralized by the addition of bisulphite The 3 types of *poliovirus are represented in the final trivalent pools as follows* Type I 3 parts, type II 1 part, and type III 2 parts

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Vaccine 555 L	III	<2	<2	<2	<2	<2	<2	<2		–	256
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* Titers as reciprocals of serum dilution
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Not done

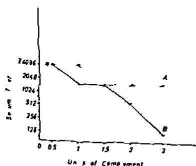


Fig 1

CF test with two different polio I antigens (A and B) in the presence of varying amounts of complement a anticomplementary

In Fig 1 is recorded the serum titers obtained employing two type I vaccines of different antigenic potency with varying units of complement The serum titer obtained with vaccine antigen *A* varied only slightly with different amounts of complement However, with antigen *B* the serum titer was found to increase with decreasing amounts of complement and when 1 5 units of complement was used the two antigens gave the same serum titer

Concentration of Polio vaccines in the Ultracentrifuge

Monovalent vaccine A sample of type III vaccine was concentrated 30 × in the ultracentrifuge with the addition of gelatin 0 06 per cent and the sedimentt was resuspended in Hanks' balanced salt solution The original material and the 30 × concentrate were titrated simultaneously

The CF antigen titer of the vaccine was 1 and the titer of the concentrated antigen was 1 32 The recovery of the antigen by ultracentrifugation was thus satisfactory No crossing with the 2 heterologous polio sera was noted either with the undiluted original or with the 30 × concentrate diluted 1 2 Similar results were obtained with type I and II The supernatant of centrifugated vaccines never gave any reaction when examined in CF tests with potent homologous or heterologous sera (9)

TABLE 4

Correlation of the Dilution of the 3 Components in the Final Trivalent Vaccine with the Required Concentration and the Endpoint Dilution of the 10 × and 30 × Concentrate for the Detection of the Component in CF Test

Component	Dilution in the trivalent pool	Required Concentration	Endpoint dilution of the concentrate for the detection of component	
			10 x concentration	*30 x concentration*
Type I	1 2	2 ×	1 4	1 16
Type II	1 6	6 ×	1 1 1 2	1 5
Type III	1 3	3 ×	1 2 1 3	1 9

Trivalent polio vaccine is in our laboratory prepared by pooling 3 parts of type I, 1 part of type II, and 2 parts of type III vaccine In the resulting trivalent vaccine the type I component is therefore present in a dilution 1 2, the type II in a dilution 1 6, and the type III in a dilution 1 3 When the trivalent vaccine was used undiluted as antigen in CF tests, only the type I component gav a positive result, as it was to be expected, while no reaction was obtained with the type II and type III components In order to be able to demonstrate these two components in the final trivalent vaccine by CF tests, it was therefore necessary to concentrate the vaccine

In Table 4 are listed the results obtained in CF tests with the trivalent vaccine concentrated 10 × and 30 × as antigen The titer of the con-

centrated antigens (endpoint dilution of antigen) was found to correspond well with the values to be expected if the concentration procedure was satisfactory e g did the 30 times concentrated antigen give a type I antigen titer of 16 which was to be expected since the type I component represents half of the volume in the trivalent vaccine

TABLE 5

Protocol of the Testing in CF of the Monovalent Vaccines and the Trivalent Pool in Concentration 10 × and 30 ×

		CF titer of monkey antiserum			CF titer of antigen		
		I	II	III	I	II	III
Type I	567	1024			1 1		
	569	1024			1 1		
	570	1024			1 1		
Type II	549		128			1 1	
	550		128			1 1	
Type III	555			256			1 1
	556			32			1 1
Trivalent Pool		256	<32	<32			
10 × concentrate		1024	128	256	1 4	1 2	1 2
30 × concentrate		1024	128	256	1 16	1 4	1 8

Each of the type I and type III components were represented in the trivalent pool in 20 liter volumes while the 2 type II components each were included in 10 liter amounts

Titers expressed as reciprocals of serum dilution

In Table 5 is presented a protocol of the CF titration of the original components of a polio vaccine as well as of the final trivalent pool and the 10 × and 30 × concentrates In the preparation of these pools the two type III components were found to differ somewhat in potency in the CF test as measured by the serum titer obtained In the 10 × concentrate of the vaccine the type III component had a titer of 1 2 while the 1 4 dilution did not react at all Since the expected titer of the type III component in the 10 × concentrate would be 1 2 1 3 it may be concluded that a concentration of an antigen by ultracentrifugation is not always quite quantitative

CF Antigen Contents of Monovalent Components during the Processing of the Polio Vaccine

In order to follow the CF antigen of the monovalent vaccines during the process of inactivation with formalin 1 4000 at 37° C samples were collected at different stages of the processing from one type II and one type III monovalent vaccine All the samples were dialyzed before used in the CF test The results are recorded in Table 6 which also shows the data from a similar study carried out with inactivation at 25° C with formalin 1 2000 (13 14)

It will be seen from the table that the formalin treatment resulted in a slight reduction of the CF antigen content for type II as well as for type III by both kinds of inactivation procedure. This drop in antigenicity was reflected in a *2–4 fold* drop in serum titer in the CF tests during the inactivation period.

Similar results (not included in Table 6) were obtained in other experiments with type I.

TABLE 6

Decrease of CF Antigen during the Inactivation of Poliovaccine as Measured by Serum Titer

Sample day	CF titer of homologous monkey serum							
	Formalin 1:4000 pH 7 temperature 3[illegible]° C				Formalin 1:2000 pH 7 temperature 25° C			
	Type II Vaccine No 611		Type III Vaccine No 619		Type II Vaccine No 612		Type III Vaccine No 64	
	1:1*	1:2	1:1	1:2	1:1	1:2	1:1	1:2
0	256§	32	512	128	128	< 32	512	< 32
1	256	32	512	64	64	< 32	512	< 32
3	256	64	1024	256				
6	128	< 32	512	64				
6+	256	< 32	256	< 32				
7					64	< 32	256	< 32
9	128	< 32	256	< 32				
12	128	< 32	256	< 32				
14					128	< 32	512	< 32
14+					128	< 32	256	< 32
25					128	< 32	128	< 32
35					64	< 32	128	< 32
56					64	< 32	128	< 32

* Dilution of antigen

§ Titers are expressed as reciprocals of serum dilution

† Sample after filtration

Anticomplementary Activity of Poliovirus Kept at 37° C with and without Formalin

In the previous experiments formalin treated polio vaccines were used as antigens in CF tests.

Another experiment was set up in order to compare the A-c activity of poliovirus suspensions kept at 37° C with and without the addition of formalin. Cell debris was removed from the infectious virus TC fluids by centrifugation but no filtration was carried out. The virus titer of the fluids was 10^{-3}, 10^{-6} and 10^{-9} per 0.2 ml for types I, II and III respectively.

The pH of the fluids was ca. *8 and was not adjusted. Formalin* was added to a final concentration of 1:4000 and the fluids were placed in a water bath at 37° C. For each virus type controls without formalin were included. *On days 0, 1, 3, 7, 9, 12* and 15 of the experiment samples were collected from the three types as well as from the controls. The

samples were stored at +4° C until used in CF tests The results are recorded in the left part of Table 7

Titration of complement in the presence of the various samples collected revealed that the formalin treated fluids became anti-C' during the first day of inactivation and remained so to some degree for at least 6 days (Fig 2, part A) By dialysation of the fluids the anti-C' effect was completely removed

TABLE 7

Decrease of CF Antigen during the Inactivation of Unfiltered Poliovirus at 37° C with Formalin 1:4000, at pH 8 and at pH 7

Sample day	CF titer of homologous monkey antiserum									
	pH 8					pH 7				
	Type I antigen 1:1	Type I antigen 1:2	Type I antigen 1:4	Type II antigen 1:1	Type III antigen 1:1	Type I antigen 1:1	Type I antigen 1:2	Type II antigen 1:1	Type II antigen 1:2	Type III antigen 1:1
0	2048*	1024	128	128	512	2048*	512	128	<32	512
1	2048	512	<128	128	128	2048	512	128		512
2						2048	512	128		256
3	1024	512	<128	128	128	2048	512	128		256
5						1024	256	128		256
7	1024	512		32	128	1024	256	128		256
9	1024	512		64	128	1024	128	128		256
12	256	<128		64	256	1024	256	128		256
15	128	<128		32	32	1024	256	256		256

* Titers are expressed as reciprocals of serum dilution

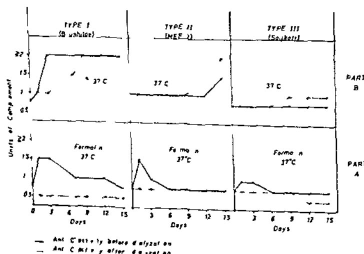


Fig 2

Anticomplementary activity of poliovirus after inactivation at 37° C without and with formalin before and after dialyzation, pH $\geq$ 8

The results obtained with heat-treatment alone (without formalin Fig 2, part B) were less regular The type I fluid thus became strongly anti-C', and this effect could not be removed by dialyzation

The decrease in CF antigen during formalin inactivation at pH 8 for 15 days was substantial, the drop in serum titer being 16-fold for types I and III and 4-fold for type II (Table 7, left part) A similar experiment was carried out at pH 7 and is recorded in the right part of Table 7 Here the loss as expressed in CF serum titer was unsignificant

From these experiments it seems that the pH of the virus suspension is important for the preservation of CF antigen during the inactivation of poliovirus

It should be added that no antigenic cross-reactions were found after 15 days of inactivation in CF tests, even when the heterologous monkey sera were used in a dilution of 1 16

DISCUSSION

The results reported here confirm observations made by others (7 15, 16) that formalin treated poliovirus can be used as antigen in CF tests However, the titer of the antigen is low, and it is therefore necessary to use the CF serum titer in order to express the relative potency of various formalin treated polio antigens

In our experiments the CF test could regularly be used to demonstrate the presence of antigenic material in the monovalent polio vaccines of all 3 types CF tests could also be employed to demonstrate the type I component in the trivalent vaccine manufactured in our laboratory

However, since types I, II and III are represented in final dilutions of 1 2, 1 6, and 1 3, respectively, in the trivalent vaccine, it was necessary to concentrate the vaccine by high speed centrifugation in order to be able to demonstrate the type II and type III antigens in CF tests In contrast to the finding by other workers (16) no CF antigen could be demonstrated in the supernates The same authors (16) found non-specific cross-reactions between the 3 types of formalin inactivated poliovirus in CF tests No such crossing was observed in the present studies

Complement fixation tests on samples removed at different stages of inactivation of poliovirus revealed that a slight loss of CF antigenic material occurred during the inactivation period This decrease varied somewhat from batch to batch for all 3 types, but the present experiment seemed to indicate that the type III antigen was slightly more vulnerable than types I and II

It is of interest that the loss in CF antigen was of the same order of magnitude whether the virus inactivation was carried out with formalin 1 4000 at 37° C or at 25° C with formalin 1 2000 (Table 6) In contrast, changing the pH to 8 (in experiments with formalin 1 4000) had a more harmful effect on the CF antigen (Table 7)

It was found that the virus fluid becomes anti C after the addition of formalin and remains so for a varying number of days the fluid again becomes non anticomplementary at the end of the inactivation period *Gard* (17) has presented evidence that the concentration of formaldehyde decreases during the inactivation period This observation together with the fact that the anti C activity can be removed completely from formalin treated poliovirus by dialyzation (Fig 2 A) points to the formalin treatment as the factor responsible for the anticomplementary activity of the vaccine Heat treatment alone (without formalin) may also render the virus fluids anticomplementary (Fig 2 part B) This effect cannot however, be removed by dialyzation

Warren et al (10) have found that polio vaccine under certain circumstances may retain its ability to fix complement after it has lost its immunizing capacity for monkeys

In the present experiments only one monovalent vaccine (type II) was found not to be able to fix complement This particular batch was also found not to be antigenic for guinea pigs

No information on the correlation of the complement fixing activity of a vaccine and the antigenic activity in humans is to our knowledge available at present However it seems reasonable to conclude that in our set up the demonstration of viral antigen in the unconcentrated monovalent polio vaccines is a useful tool in the production of polio vaccine as it will allow the exclusion of inferior batches of monovalent vaccine without antigenicity testing in animals

SUMMARY

The content of complement fixing antigen in monovalent polio vaccines as produced in our laboratory has been found sufficient to react with monkey hyperimmune sera as well as with guinea pig sera from routine antigenicity tests The tests were found to be type specific

While the m [illegible] hout concen [illegible] the trivalent [illegible] tigen content [illegible] to decrease slightly during inactivation at pH 7

It is suggested that CF tests constitute a useful supplement to other antigenicity tests on formalin inactivated polio vaccines particularly as regards preliminary testing of monovalent vaccine batches

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ETIOLOGY OF RESPIRATORY TRACT INFECTIONS IN MILITARY PERSONNEL

I Virological Findings

By

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L PHILIPSON and T WESSLÉN

Received 25 ii 61

During recent years considerable study has again been devoted to respiratory tract infections For the most part, they have been considered to be caused by viruses or at least viruses are thought to be the primary etiological agents (1) but the role of the bacteria in these infections is still under dispute

Tissue culture methods have provided for the discovery of several new' viruses, which have been associated with respiratory tract infections Some types of adenoviruses seem to be definitely associated with these infections in military personnel and in civilian population groups although the incidence of adenovirus infections in civilians seems to be far less than that in military personnel (10)

Another group of viruses associated with respiratory tract infections has also been demonstrated These agents have shown biological resemblance to parotitis virus and Newcastle disease virus The group has been called the parainfluenza viruses and includes three different types (2)

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Finally several agents belonging to the Echo-group or viruses not differing biologically from members of this group have also been associated with respiratory tract disease (1) A number of virus strains associated with the common cold and not yet classified have also been found (24)

Research on the etiology of respiratory tract infections in military personnel has been carried out in Sweden with more or less intensity during the last decades In the middle of the thirties the interest was concentrated on pneumococcal lobar pneumonia (8, 13) Later the use of sulfonamides in prophylaxis and therapy was for some years the main topic (14) During and after the world war II the studies were

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17 *Gard* … *Theoretical and Practical*

MATERIAL AND METHODS FOR VIROLOGICAL EXAMINATION

Collection of Specimens

Throat The virological specimens from the throat were taken by cotton swab from the tonsil and hypopharynx region The swabs were immediately placed in tubes with Parker 199 medium with 5 per cent horse serum and 1000 units of penicillin and 1000 mcg of streptomycin/ml These tubes were subsequently frozen in dry ice thermosflasks and transported to the virological laboratory where they were frozen at —60° C for not longer than a fortnight before analysis

Faeces These specimens were taken by rectal swabs of cotton which had been soaked in 50 per cent sterile glycerin immediately before use They were also placed in the Parker medium and frozen in dry ice before transportation to the laboratory where they were stored at —60° C for not longer than 14 days

Blood The blood was taken by ven puncture about 25 ml was collected each time The serum was pipetted off after coagulation for 12 hours at +4° C and centrifugation It was then frozen at —20° C in tubes containing about 4 ml of serum each

Tissue Cultures

Monkey kidney Trypsinization of Rhesus or Cynomolgus monkey kidney was

was used The cells were
The maintenance medium

Hemadsorption Test

The hemadsorption test was carried out according to the modification of *Vogel & Shelokov*'s technique (21) reported by *Chanock et al* (4)

Virus Isolations

All virological specim
tissue
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d
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specific rabbit sera

Antibody Analysis

All sera were tested for complement fixing antibodies against influenza virus A B and C, adenovirus parotitis parainfluenza 1 2 and 3 and ornithosis Further the Paul Bunnell reaction for mononucleosis was ca

Some of the viral antigens showed anticomplementary activity especially those

concentrated on virus research, especially with influenza virus. Influenza in military personnel has been caused by influenza B in 1943 (16), by influenza A in 1944 (15) by influenza B in 1946 (16), by influenza A, type A1, in 1947 (16). In 1950 sporadic cases occurred during the summer and a fairly extensive epidemic followed during the early part of 1951. The isolated strains were related to 1947 A1 virus (19). In 1957 influenza virus A2 of the Asian type caused a wide spread epidemic also in Sweden (12).

Adenoviruses have been studied in some small epidemics mostly in children (11).

An attempt to vaccinate military personnel against influenza was made in 1947 but with the same negative results (6) as in other parts of the world. This was presumably because the vaccine did not correspond to the virus type, which caused the epidemic. A new attempt with greater success was made with the strain A 2 just at the start of the epidemic of Asian influenza (9) in 1957.

Although considerable literature has been gathered during recent years concerning the role of the various viruses of the respiratory tract in epidemics or in sporadic cases over a period of many months, studies considering bacteria and viruses at the same time are sparse.

The aim of the present investigation is to follow the respiratory tract infections of military personnel over a number of years. It is assumed that the etiological agents will change from one infection season to the next, and the hope is to get a fairly broad analysis of this group of infections in a long term programme.

In a first series of papers the results from three periods, the winter and spring of 1959, the autumn of 1959, and the winter of 1960, will be reported. The present first paper deals with the virological findings, a second will give the bacteriological results and in the third paper an analysis of the clinical observations will be undertaken in the light of the virological and bacteriological results.

PLAN OF STUDY

The investigation was carried out at a military camp at Uppsala. The number of conscripts varied between 580 and 989 partly due to the fact that they were on duty elsewhere in Sweden for various length of time when they could not be examined.

All conscripts who were reporting for respiratory tract infections at the hospital of the camp were examined by a group of one physician and one assistant from the laboratory. They visited the camp every morning, examined the conscripts with respiratory tract infections and took the specimens. Disease histories and symptoms were registered and the following specimens were taken.

Specimens from the nose and throat for bacteriological and virological examination were taken at the first visit and repeated after five days.

Faeces specimens were taken for virological examination at the same intervals.

Blood samples were collected at the first visit and repeated twice at ten day intervals.

The course of the disease was recorded each time a specimen was taken. The whole study was carried out in close collaboration with the head physician of the establishment. Only cases with a complete set of specimens are reported, though however only two specimens of blood were procured in 20 per cent of the cases.

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Tissue Cultures

Monkey kidney Trypsinization of Rhesus or Cynomolgus monkey kidney was carried out according to methods described before (22) The maintenance medium was Parker 199 solution with antibiotics (100 units and 100 mcg/ml of penicillin and strept

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Antibody Analysis

All sera were tested for complement fixing antibodies against influenza virus A, B and C, adenovirus parotitis parainfluenza 1 2 and 3, and ornithosis Further the Paul Bunnell reaction for mononucleosis was carried out on each serum All specimens from the same case
the antibody titres were r
The complement fixati
gen and serum respective
viously (18)

Some of the viral antigens showed anticomplementary activity especially those

produced in tissue cultures This was reduced by the addition of inactivated (56° C 30 min) guinea pig serum and incubation for 1 hour at 37° C according to the technique described by *von Zeipel* (23)

Antigens

Paul-Bunnell test This reaction was carried out according to the original method (17) When the titre exceeded 1/40 the Davidsohn adsorption test was carried out (5)

Influenza A, B and C-antigens The antigen was produced as a membrane antigen in egg for ... homogenization of the membran ... supernate was used as antigen ... ids from chick embryos infected with the strain 1233 Taylor

Parainfluenza antigen The three type strains HA2 Ca and HA1 were cultivated on monkey kidney cultures with Parker 199 as maintenance medium When the cytopathic effect was maximal the tissue culture fluids were harvested and centrifuged at 2000 r p m for 15 min After dialyzing the supernatant against veronal buffer pH 7 5 it was used as the antigen

Parotitis antigen The antigen used was a virus antigen obtained by Sharples centrifugation of infected allantoic fluid from chick embryos

Adenovirus a ... from tissue culture f ... per cent methanol ... this procedure

Ornithosis antigen The Squibb "Lygranum C F " antigen was used Control antigens were tested concurrently with the lygranum antigen in those sera positive against the lygranum antigen

RESULTS

The average number of conscripts and the number of cases during the various periods are reported in Figure 1 and 2, top part The total number of cases was 221, of which full serological testing could be carried out according to plans in 208 cases In 13 cases an inadequate number of blood samples was received

The results of virus isolations are presented in Figure 1 Figure 2 shows cases with a fourfold increase in antibody titre The figures give the following general picture of the occurring infections

Influenza A The influenza strains of February 1960 were of the Asian type The first occurred on February 2nd and the epidemic came to an end on March 3rd 66 cases of respiratory tract infections were hospitalized during this time and 41 were diagnosed as influenza A (4 6 per cent of the total force)

Influenza B During the period 12 1 27 2 1959, increased titres for influenza virus B were found in altogether 44 cases The cases had mostly the character of common cold and no influenza B virus was isolated

Influenza C Increased antibody titres for influenza virus C occurred in two cases In one case this virus was the only detectable cause of the disease, in another case antibodies for influenza C occurred simultaneously with influenza B-antibodies

Parainfluenza No viruses of the parainfluenza type were isolated A few cases showed an increase in antibody titres, but the etiological significance of these findings is uncertain



Fig 1
Number of cases and isolations of viruses during the winter of 1959, the autumn of 1959 and the winter of 1960
□ number of cases
■ simultaneous isolations of two different viruses

Adenovirus type 3 During the outbreak of influenza B in January and February 1959, mentioned above, adenovirus type 3 was isolated in 24 cases, an increase of antibody titre occurred in four of these cases, and in addition, four cases showed an increase in antibody titres without positive virus isolations In four cases a simultaneous increase in antibodies could be shown for adenovirus and influenza B

Adenovirus type 7 Adenovirus type 3 and 7 were both isolated in one case in January, 1959 In September, 1959, adenovirus type 7 was isolated in two cases, and antibodies were found in due course in these cases During the epidemic of influenza A in January and February, 1960 this virus was isolated in sixteen cases, and an increase in antibody titres occurred in eleven of these cases An increase in antibody titres without positive virus isolations occurred in ten cases An increase in antibody titres for both adenovirus 7 and influenza virus A was found in nine cases

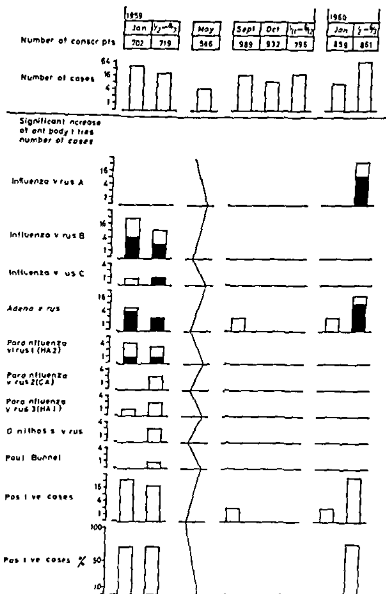


Fig 2

Number of cases and occurrence of antibodies showing at least a fourfold increase in titres

☐ number of cases and increase in titre of antibodies for one virus
■ increase in titres of antibodies for at least two different viruses

Miscellaneous viruses In the autumn of 1959, poliovirus type 1 was isolated from six faecal specimens No case of clinical poliomyelitis was, however, diagnosed Two strains of Herpes simplex virus was isolated in October, 1959 and one strain in January, 1960, without any clinical manifestations

The antibody titrations revealed a fourfold increase for ornithosis virus in two cases in February, 1959 A positive Paul-Bunnell test was also found during the same month

It is seen from the Figure 2 that the frequency of positive cases—

which here means cases showing at least a fourfold increase in antibody titres for the virus in question—is for January, 1959 71 per cent, for February, 1959 71 per cent and for February, 1960 77 per cent The average percentage of the whole material was 57 per cent

DISCUSSION AND SUMMARY

From the virological point of view, the material was dominated by the outbreak of influenza B in 1959 and the epidemic of influenza A in 1960 The latter spread all over Sweden and had a fairly mild course The simultaneous occurrence of adenovirus type 3 during the influenza B and adenovirus type 7 during the influenza A epidemic is of interest, as it indicates that respiratory tract infections caused by viruses might be mixed infections They are then dominated by the virus which is most aggressive in these two outbreaks, the influenza viruses A further complication in the analysis of these infections involves the role of the bacteria which will be dealt with in the following two papers A more extensive *discussion of the results will be presented in paper III*

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24 Leading article Common Cold Viruses Lancet *1* 266 1960

FROM THE DEPARTMENTS OF BACTERIOLOGY AND VIROLOGY,
UNIVERSITY OF UPPSALA, SWEDEN

AETIOLOGY OF RESPIRATORY TRACT INFECTIONS IN MILITARY PERSONNEL

2. Bacteriological Findings

By

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L. PHILIPSON and T. WESSLÉN

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Concerning the aetiology of acute respiratory infection, especially during recent years, attention has largely been focussed on viruses, as has been pointed out in the introductory article of this series (5). Among the bacteria, however, the beta streptococci and their importance in acute tonsillitis and exudative pharyngitis have been the object of detailed studies: the incidence of streptococcal infections having varied between 4 and 8 per cent in such investigations. Other common pathogenic bacteria present in the respiratory tract, including Staphylococcus aureus, pneumococci, and H. influenzae, have been ascribed relatively minor importance in causing acute respiratory infections. In the comprehensive investigations carried out in 1947 by the Commission on Acute Respiratory Disease (4) the correlation between the expected and observed numbers of carriers was poor with regard to the various species of respiratory-passage bacteria in connexion with re-infection. The family studies of *Brimblecombe* and his colleagues (1) showed a similar trend, but they made the interesting observation that the incidence of pneumococci increased during attacks of "common cold", and that the outbreaks of this infection could be followed with the aid of pneumococci. It was found in a Swedish investigation carried out during the 1940's at a number of day nurseries (8) that a marked increase in certain pneumococci and beta streptococci took place at periods during which there was a particularly high incidence of respiratory infection; this was especially true of children aged under 3 years.

Certain recent investigations also suggest that the part played by bacteria in respiratory infection is possibly being underestimated. *Ritchie* reports two studies which indicate this. In the one (11) he used autovaccine, and in the other (12) antibiotics, prophylactically. In both series he obtained a reduced incidence of respiratory infection. *Burke* (2) showed that children given sulphadimidine prophylactically before tonsillectomy ran less risk of bacterial infection. These series must be

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TABLF 1

Distribution of the Various Bacteria Isolated from 222 Cases of Upper Respiratory Infection at a Military Camp at Uppsala during the Winter and Autumn of 1959 and the Winter of 1960

	Staph aur %	Beta strep-tococci %	H influen-zae %	Pneumo-cocci %	Meningo-cocci %
Winter 1959					
Nose and throat	7	2	0	3	1
Throat	22	15	3	6	0
Nose	7	1	10	12	6
Negative	64	82	87	79	93
Total positive specimens	36	18	13	27	7
Autumn 1959					
Nose and throat	10 6	6 4	–		
Throat	25 5	31 9	6 4	4.3	2 1
Nose	17 0		2 1	4.3	2 1
Negative	46 8	61 7	91 5	91 5	95 8
Total positive specimens	53 2	38 3	8 5	8.5	4.2
Winter 1960					
Nose and throat	12 2	2 7	4 1	1 4	–
Throat	40 5	24 3	4 1	4 1	–
Nose	12 2		9 5	8 1	1 4
Negative	35 1	73 0	82 4	86 5	98 6
Total positive specimens	64 9	27 0	17 6	13 5	1 4

RESULTS

The incidence of bacteria isolated from the respiratory passages during the three periods of investigation can be seen from Table 1 and Fig 1 Only those organisms are recorded which were isolated from swabs taken on the first examination, before they had been in contact with the hospital environment The cultures from the second set of swabs are affected by treatment and possibly also by nosocomial infection Bacteria negative swabs were obtained from 32 7 per cent of cases during winter 1959, from 10 7 per cent during autumn 1959, and from 16 2 per cent during winter 1960

No particular organism predominated in the positive swabs, but Staph aureus was most commonly present in 36 per cent, 53 2 per cent and 64 9 per cent of cases during the respective periods The corresponding figures for beta-streptococci were 18 per cent, 38 3 per cent and 27 per cent H influenzae was roughly equally common during each period, viz 13 per cent during winter 1959, 8 5 per cent during autumn 1959, and 17 6 per cent during winter 1960 It may be noted that meningococci were isolated during all three periods, the figures being 7 per cent, 4 2 per cent and 1 4 per cent respectively, no case of meningi-

regarded as being to some extent selected, however, and few large investigations have been published on the aetiology of respiratory infection studied from both virological and bacteriological aspects As stated in the introductory paper of this series, therefore, a study of this nature was therefore decided upon, taking into consideration the part of the bacteria both as primary aetiological factor and as complicating a virus infection

MATERIAL

The investigations were carried out at a military barrack near Uppsala over three long continuous periods during the years 1959 and 1960 For details of the planning of study during the periods in question the reader is referred to the first paper of the series Results of the bacteriological investigations were obtained in a total of 222 cases

METHODS

Data concerning the extent and technique of specimen taking may be found in the preceding paper (5)

Culture All samples were cultured on the following media

(1) Meat infusion agar with 5 per cent sheep s blood 2 plates
(2) Meat infusion agar with 5 per cent sheep s blood and gentian violet (1/750 000)
(3) Phenol mannite agar (Chapman 3) containing 7 5 per cent NaCl
(4) Meat *infusion* agar with 6 per cent haematin
(5) Meat infusion broth containing 6 5 per cent NaCl for enrichment of staphylococci
(6) Enrichment *medium as described by Holmes & Lermit (6) for isolating* beta streptococci

All cultures were incubated at 37° C and were read after 24 and 48 hours Media 1 was used for both aerobic and anaerobic culture and media 2 and 4 were incubated in a 10 per cent carbon dioxide atmosphere After 24 hours growth in the enrichment broth the cultures were plated out on media of types 1 and 2 On *reading the species of bacteria were noted* and colonies suspected of being pneumococci beta streptococci H influenzae staph aureus or meningococci were further tested as follows

Pneumococci were confirmed by means of their optochin sensitivity and when sensitive typed by *Neufeld* s capsule swelling method The optochin test was carried out by a paper strip method

Streptococci were tested for the presence of soluble haemolysins and the sensitivity to bacitracin was examined by *Maxted* s technique (9)

Beta streptococci were typed by Dr Ingemar Juhlin Department of Clinical Bacteriology Allmanna sjukhuset Malmo

H Influenzae were confirmed by the positive satellite phenomenon

Staph aureus were verified by the coagulase test performed on a glass slide Phage typing was not carried out

Meningococci were confirmed by fermentation with dextrose maltose and laevulose

The following serological investigations were performed

Antipneumolysin testing (API) was carried out by a method described by Tunevall (14)

Antistreptolysin determination (AST) was *done by a modification of Ibsen* s method (7) The readings were made as described by Packalen and Bergquist (10) for the determination of antistaphylolysin

Antistaphylolysin testing (ASTA) *was performed by the method* of Packalen and Bergquist (10)

Antibodies against influenzae bacteria were estimated by a complement fixation reaction described by *Tunevall* (13)

TABLE 2

Result of Typing of beta-Streptococci

Beta haemolytic streptococci		Number of cases	
Group	Type	1st visit	2nd visit
Winter 1959			
A	4, 44 46	1 + 1*	1*
	4 46, 29	–	1
	3, 44	2	–
C	21	2	1
E	–	1	–
G	16	3 + 1*	1*
H	–	1	–
L	–	1	–
Non typable	–	5	1
Total		18	5
Autumn 1959			
A	1	1	
	1, 46	1	
	5 9, 12 27, 44	1	
	9	2	
	22	1	
C	21	4 + 1*	1*
G	–	1	
	16	2	
Non typable		3	1
Total		17	2
Winter 1960			
A	1	1	
	3	1*	1*
	5 27	1	
	9	2	
	10	2	1
	12	1	
	22	1	
C	21	2	1
F	–	1	
G	–	1	
	16	1*	1 + 1*
Non typable		3	1
Total		17	6

* Denotes that the same type of streptococcus was isolated from the same patient on both occasions

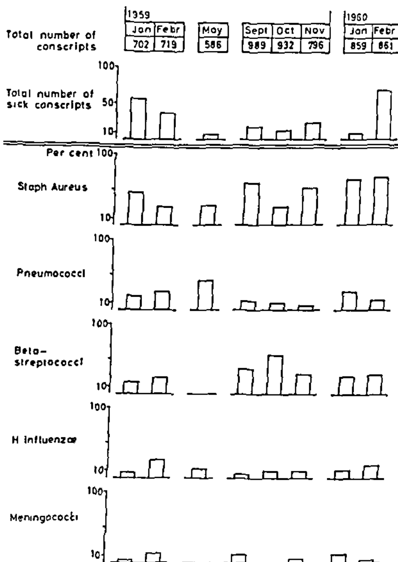


Fig 1

Incidence of various bacteria per cent of conscripts with acute respiratory infection during winter and autumn 1959 and winter 1960

tis occurred, however Fig 1 shows no marked concentration of bacteria at any particular time, suggestive of an epidemic due to the organism in question, the slight peak of beta-streptococci in October 1959 might possibly suggest such an outbreak

In some cases it was possible to isolate two or more species of bacteria from the same individual, the most common finding being *Staph aureus* in combination with some other organism

Typing of beta-streptococci The results are collected in Table 2 As can be seen, typing proved possible in a satisfactory proportion of cases No epidemic concentration of any particular type of streptococcus occurred on any occasion Notable, too, is the scanty incidence of group-A streptococci *during winter 1959 As a rule beta-streptococci, when pre-*

respiratory passages, but untreated, are included As can be seen, the effect of penicillin upon penicillin-sensitive organisms was good This was particularly true of beta-streptococci and meningococci, which were almost completely eliminated The results were less good with pneumococci, and particularly striking are the 10 cases in which despite treatment pneumococci were isolated from the second swab only Staph aureus was eliminated in a surprisingly large number of cases, but this must be viewed in the light of the nature of the series and tests on a selected number of strains showed that all were penicillin sensitive The findings concerning H influenzae were interesting During winter 1959 they were still being isolated from 5 out of 10 cases after treatment, and in 5 cases they were found first in the second swab despite treatment It is of course difficult to establish whether bacteria isolated in the second of two swabs taken at an interval of 5 days might be an expression of new infection or whether they were due to the increased yield that was always to be counted on when several samples were taken Both these factors were probably at play, but especially concerning H influenzae nosocomial infection might have been relatively common

TABLE 4

Bacteriological Findings in 137 Cases Treated with Penicillin and in 80 Cases not Treated with Penicillin

Type of bacteria	Bacteria isolated					
	Only at 1st visit		Both at 1st and 2nd visit		Only at 2nd visit	
	Treated	Not treated	Treated	Not treated	Treated	Not treated
Winter 1959						
N mening	6		1	–	–	1
Pneumococci	13	4	4	–	5	2
Beta strep	13	1	1	1	1	2
H influenzae	5	2	5	2	5	1
Staph aureus	19	5	7	4	2	5
Autumn 1959						
N mening		1	–	–	1	
Pneumococci	2	1	–	–	3	1
Beta strep	15	1	–	–	1	–
H influenzae		2	–	–	2	1
Staph aureus	13	7	4	4	4	2
Winter 1960						
N mening	1		–	–	–	
Pneumococci	6	2	1	–	2	–
Beta strep	14	3	2	1	–	3
H influenzae	6	6	1	2	8	2
Staph aureus	23	19	11	13	3	1

sent were isolated from the first swabs, but in 10 cases they made their first appearance at the second sampling In 7 cases beta-streptococci were isolated from both first and second swabs, more than one type of streptococcus being present in two cases

Typing of pneumococci The results are collected in Table 3 A surprisingly large number of organisms proved untypable None of the typable strains tended to predominate In 7 cases they were first isolated from the second swabs, and in 6 cases they were isolated from both first and second swabs

TABLE 3
Results of Typing of Pneumococci

Pneumococci	Number of cases	
Type	1st visit	2nd visit
Winter 1959		
9	1	
11	1	
15 23	1	1
17	2	
19		1
20	1	
Non typable	11 + 4*	3 + 4*
Total	21	9
Autumn 1959		
18		1
22		1
33	1	
Non typable	3	2
Total	4	4
Winter 1960		
9	1	
40	3	
Non typable	5 + 1*	1 + 1*
Total	10	2

* Denotes that the same type of pneumococcus was isolated from the same patient on both occasions

H influenzae Strains of this organism were not typed It is worthy of note that H influenzae was isolated from the second swabs only in a large proportion of cases (37 per cent)

Results of treatment Penicillin treatment, with the usual dosage, was employed in a large number of cases A broad-spectrum antibiotic was used in only one case The results of treatment are shown in Table 4 For purposes of comparison subjects with latent pathogens in the

Influenza A infection was also present in 8 of the 14 cases of streptococcal infection Pneumococci too were common during this period, being present in 10 cases It is odd that pneumococci could not be isolated in more than half of the cases in which there was significant increase in the antipneumolysin titre

DISCUSSION AND SUMMARY

The results of the bacteriological and serological investigations carried out in this study showed no important divergencies from those previously reported or from what might be anticipated in a series such as this With the exception of beta streptococci during September and October 1959, the incidence of latent pathogenic bacteria was no higher than is usual among healthy National Service men The finding of meningococci during both periods of investigation indicates that swab-taking transportation, and culture technique were satisfactory The [illegible]

October 1959 however, was matched by a high incidence of significant rises in titre The same was found during the influenza A outbreak in February 1960 Most of the streptococci isolated during these periods belonged to group A, but no particular type predominated It was not unusual to find significant rises in antibody titre in the absence of the corresponding pathogenic bacteria in the respiratory passages, and this was rather difficult to explain It was also noteworthy that smaller rises in titre occurred within the different systemts without apparently being accompanied by corresponding clinical infection This might possibly be due to unspecific response to some other acute infection

The results of penicillin therapy were in general good when the organisms were penicillin sensitive (*e g* beta streptococci and meningococci) In the case of H influenzae the results were less good, and nosocomial infections might have been involved It is not intended to discuss here the clinical significance of the findings, as this will be done in the final article of the series Briefly, however, it might be said that a concentration of primary beta streptococcal infections took place during autumn 1959 and winter 1960 and bacteria such as pneumococci and H influenzae were *probably also* of primary aetiological significance Apart from this concentration of primary streptococcal infections, bacterial infections were chiefly secondary to primary virus infections in the outbreaks of acute respiratory disease

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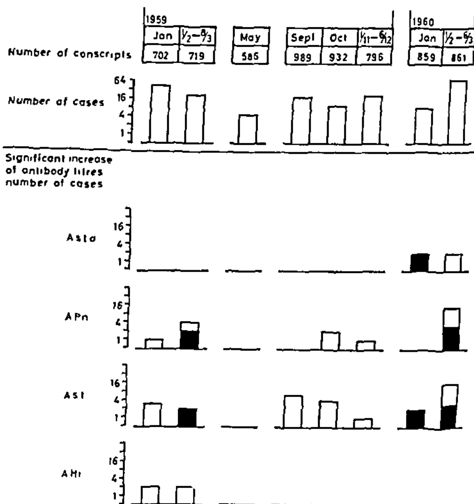


Fig 2

Incidence of significantly raised titres against antistaphylolysin (Asta) antipneumolysin (Apn) antistreptolysin (Ast) and complement fixating antibodies against *H* influenzae among sick conscripts

□ = rise in titre against a serological titre system
■ = simultaneous rise in titre against another serological system in addition

Serological tests Up to the present Ast, Asta, and APn estimations have been done on the whole series AHi estimations were carried out *only on the winter-1959 series* *The requirement for significant rise* in titre was, for lysin systems more than a doubling in titre, and for AHi increase by at least four times With AST, however, a slightly smaller increase was sometimes accepted *in clinically unequivaocal* cases of streptococcal tonsillitis (See paper 3)

Fig 2 *shows the distribution of the serologically proven cases over* the various months There was a striking *AST* peak *of 10 cases during* September and October 1959, beta-haemolytic streptococci were isolated from the throat in all of these cases

The period February March 1960, when influenza *A* was common, was also characterized by a high incidence of streptococcal infection

FROM THE DEPARTMENT OF BACTERIOLOGY, UNIVERSITY OF UPPSALA
(HEAD: PROFESSOR GUNNAR LÖFSTRÖM)

A STUDY OF THE PATHOGENICITY OF STAPH. AUREUS ISOLATED FROM DUST AND HOSPITAL ENVIRONMENT

By

G. LAURELL and H. TOTH GYULAI

Received 2 iv 61

The part played by staphylococci in nosocomial infections is well known, and these organisms are probably responsible for the majority of severe septic lesions encountered in hospitals at the present time. A very large number of investigations concerning staphylococcal infection have been published during recent years. One of the aspects of this wide field is the elucidation of the commonest mechanisms of transmission of infection, a matter about which opinions still vary. Some authors stress the significance of dust and air in transmitting infection, whereas others believe that direct and indirect contact between carriers among patients and staff is of greater importance. In attempts at prophylaxis, therefore, attention has been focussed on these different modes of infection in turn.

A condition for judging the virulence of staphylococci isolated from a source other than a focus of infection is that its pathogenicity can be established. This is true of all nosocomial infective agents, and with regard to haemolytic streptococci, for example, it has been shown in experiments on volunteers that dustborne streptococci have lost their pathogenicity (14). During recent years pathogenicity tests have also been carried out on staphylococci (1, 7, 10, 12, 15, 16), but these have been frustrated by lack of knowledge of the factors determining the virulence of these organisms. It has been claimed to have been shown with the aid of various biochemical methods, sometimes in combination with immunological techniques, that staphylococci isolated from the respiratory tract of healthy carriers have lower pathogenicity than staphylococci isolated from patients with clinical infection (9). Certain authors also maintain that their results indicate that staphylococci isolated from the hands of persons such as hospital staff are less [illegible]ous as a [illegible] in the re[illegible] authors [illegible] in pathogenicity can be found either with biochemical or immunological methods between staphylococci of the same type isolated from different sites (17).

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The part played by staphylococci in nosocomial infections is well known and these organisms are probably responsible for the majority of severe septic lesions encountered in hospitals at the present time A very large number of investigations concerning staphylococcal infection have been published during recent years One of the aspects of this wide field is the elucidation of the commonest mechanisms of transmission of infection a matter about which opinions still vary Some authors stress the significance of dust and air in transmitting infection, whereas others believe that direct and indirect contact between carriers among patients and staff is of greater importance In attempts at prophylaxis, therefore attention has been focussed on these different modes of infection in turn

A condition for judging the virulence of staphylococci isolated from a source other than a focus of infection is that its pathogenicity can be established This is true of all nosocomial infective agents, and with regard to haemolytic streptococci, for example, it has been shown in experiments on voluteers that dustborne streptococci have lost their pathogenicity (14) During recent years pathogenicity tests have also been carried out on staphylococci (1 7, 10 12, 15, 16) but these have been frustrated by lack of knowledge of the factors determining the virulence of these organisms It has been claimed to have been shown with the aid of various biochemical methods sometimes in combination with immunological techniques, that staphylococci isolated from the respiratory tract of healthy carriers have lower pathogen[illegible]

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authors no certa[illegible] [illegible]rence in pathogenicity can be found, either with biochemical or immunological methods between staphylococci of the same type isolated from different sites (17)

The present investigation was designed to compare staphylococci isolated from dust and objects with organisms originating from patients with clinical infection and from the respiratory tracts of healthy carriers

MATERIAL

Three patients under treatment at University Hospital Uppsala for staphylococcal infection were selected for the purposes of this investigation because they were judged to be 'dangerous carriers' of nosocomial infection

Case 1 11-year old girl from the Department of Paediatrics with infected generalized eczema

Case 2 21 year old man from the Department of Plastic Surgery with extensive infected burns

Case 3 38 year old man from the Department of Thoracic Surgery with pulmonary tuberculosis treated by operation During the postoperative period the wound became infected and empyema resulted

All three patients were treated in isolation rooms at the respective departments

Sampling and Culture

Specimens were taken from the nose and throat in all three cases Ordinary throat and nasopharyngeal swabs were used Specimens from infected skin lesions in cases 1 and 2 were taken by means of a throat swab which was first moistened with broth and then rubbed over the area to be examined All swabs were placed in agar slope tubes and transported as quickly as possible to the laboratory Samples of empyema pus were taken in case 3

Environment

Comprehensive investigations were made in all three cases samples being taken from 10 different sites in each patient's room (the objects examined varied slightly from case to case) Swabs were taken from the hands of two of the patients and in case 3 also from the hands of one of the nurses attending him An ordinary throat swab moistened with broth was rubbed over a given surface of the object to be examined 6 strains of staphylococci were plated out from the initial culture from each object viz 60 strains from each patient The strains were first tested for ability to form coagulase and phosphatase Tests for antibiotic sensitivity and a test for the number of diffusible antigens (*Ouchterlony*) were then undertaken in those that were positive

Dust

Samples were taken with sterile squares of flannelette that had first been washed to remove any bactericidal substances A certain area of the floor was wiped with one of these cloths which was then placed in a sterile flask in which it was stored at room temperature but shielded from sunshine until culture could be undertaken All samples from the sick room were taken on the same occasion for each individual patient 15 samples were taken from different parts of the room and cultures of these samples were repeated once weekly The observation period was therefore 15 weeks When possible 15 strains were plated out from each culture This was often impossible however especially during the final weeks when only a few colonies developed The primarily isolated strains were tested for coagulase production and those that were positive were then identified by determining their resistance pattern for antibiotics and diffusible antigens

Media

Ordinary broth 5 per cent sheep's blood agar plates and phenol mannitol agar plates with 7 5 per cent sodium chloride (*Chapman*) were used for all cultures The plates were incubated for 48 hours at 37° C and the phenol mannitol agar plates were kept at room temperature for a further 3 days before examination The swabs were placed in tubes containing ordinary broth If the primary plates showed no bacterial growth secondary culture was carried out from the broth tubes

T› prepare cultures ‹f the dust 30 ml of br‹th was placed in a flask c‹ntaining a flannelette square and vig r‹usly shaken Ab ut 1 ml ‹f this broth was streaked ont › plates which were all‹wed to dry for 30 minutes in a thermostat with the lid open and were then incubated and rea l off as above

Strains were selected from the primary culture f r continued investigati ›n on the l asis of the appearance and pigment formation of the colonies

The strains isolated were then sul jected to a series of biochemical and sero logical tests phage typed and tested for pathogenicity in mice The following techniques were employed

Coagulase Coagulase formation was estimated by the tube culture method

Phosphatase Barber & Kuper s method (2) using plates containing phen ›lphtha lein phosphoric acid was employed After incubati‹n for 24 hours at 37° C the plates were exposed to NH_4OH gas when phosphatase-forming c‹l nies became pink

Staphylokinase Christie & Wilson s (3) fibrin containing plates were used A clear zone round the colony was counted as a positive reaction

Haemolysin pattern The production ‹f alpha beta and delta lysin was deter mined by means of the antitoxin strip meth l described by *Elek & Levy* (7) The

Sensitivity *test for antibiotics* The diffusion method worked out by *H Ericsson et al* was used (8) Strains designated resistant were th‹se not inhil ited by the f ll wing concentrations Sulphonamide 6 mg% ml penicillin 2 IU/ml erythro mycin 4 γ ml streptomycin 12 γ/ml tetracyclines 4 γ/ml chl‹romycetin 12 γ/ml and novobi‹cin 16 γ/ml Strains inhibited by these concentrations are termed sen sitive

Phage typing This was carried out to a limite l extent by dr *G Wallmark* State

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The central basin was first filled with *Bur*

serum (1 000 U/ml) and the plates were incubated for 24 h urs in the refrigerator for diffusi‹n The perip heral basins were then in‹culated with the strains to be examined The substrate f r the basins was brain heart in fusi n br‹th (Difc›) with 0.3 per cent agar The plates were next incul ated for 48 hours at 37° C

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RESULTS

General characteristics of type strains The most important features of type str uns from the 3 patients are shown in Table 1 These strains were isolated from the actual foci of infection As can be seen, all strains are characterized by high activity and all form a number of extracellu lar enzymes They differ with regard to resistance pattern for antibio tics phage type and diffusible antigens Strains from case A were

TABLE 1

General Characteristics of Strains Isolated from Foci of Infection

Case	Coagulase	Phosphatase	Staphylokinase	Haemolysin Pattern	Number of precipitation lines	Alpha haemolysin titre Reciprocal value	Hyaluronidase titre Reciprocal value	Phage type and group	Resistance Pattern								
									Sulphonamide	Penicillin	Erythromycin	Streptomycin	Tetracycline	Chlortetracycline	Oxytetracycline	Chloramphenicol	Novobiocin
A Eczema	+	+	+	α δ	9	32	128	6/7/47/53/1034 Group III	○	●	○	○	○	○	○	○	○
B Burn	+	+	+	α δ	7	64	64	54/77/819/1034 Group III	●	●	○	●	●	●	●	○	○
C Empyema	+	+	+	α δ	8	64	32	NT, 1000 × RTD 53/54/70/73/75/77/819/1034 Group III	●	●	●	○	●	●	●	●	●

NT denotes non typable in test phage
1000 × RTD denotes typable in 1000 reaction test dose
● = resistant ○ = sensitive

TABLE 2

Result of Comprehensive Biochemical Tests of 75 selected Strains from Dust

	Number of strains	Coagulase +	Phosphatase +	Staphylokinase +	Haemolysin pattern				Alpha haemolysin titre Reciprocal value				Hyaluronidase titre Reciprocal value			
					αβ	αδ	βδ	αβδ	16	32	64	128	16	32	64	128
A	21	21	21	21	1	17	–	3	7	12	2		3	8	7	3
B	27	27	27	27	–	24	2	1	–	10	14	1	6	8	9	4
C	27	27	27	27	–	27	–	–	–	11	14	2	3	13	10	1

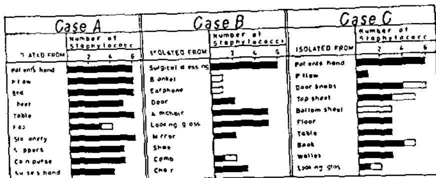


Fig 1

Result of identification studies of 114 coagulase and phosphatase positive strains isolated from different objects in the three dangerous carriers

■ denotes coagulase positive staphylococci identical to the original infective strain

□ denotes coagulase positive staphylococci not identical to the original infective strain

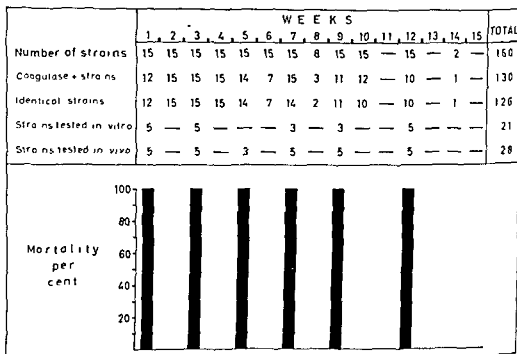
sensitive to all antibiotics but penicillin, strains from case B showed a not uncommon hospital resistance pattern, and strains from case C were characterized by exceptionally high resistance and were sensitive only to streptomycin All strains reacted to Group-III phages *Strains* from case C proved difficult to type, and reacted only with concentrated phages

Concerning diffusible antigens giving visible precipitation lines against Wood-46 serum, the figures were, for strain A 9 lines, strain B 7 lines, and strain C 8 lines In the account that follows, the terms *original strain* and infection strain always refer to the strains isolated from the focus of infection in the respective patients

Environment test Altogether 180 strains of staphylococci were isolated Of these 180 strains 114 were coagulase positive All of these were also phosphatase positive The results of identification tests by means of resistance pattern for antibiotics and diffusible antigens for these strains are shown *in Fig* 1 As can be seen, no fewer than 100 of these 114 strains were identical with one of the strains isolated from the clinical infection concerned Calculated in relation to coagulase positive strains, 52 out of 53 (98 2 *per cent*) were identical in case A, in case B the figures were 21 out of 24 (83,4 per cent), and in case C 27 out of 37 (73 per cent) were identical

In all three cases strains were isolated from the respiratory passages that in no way differed from the strain isolated from the focus of infection in the respective patient

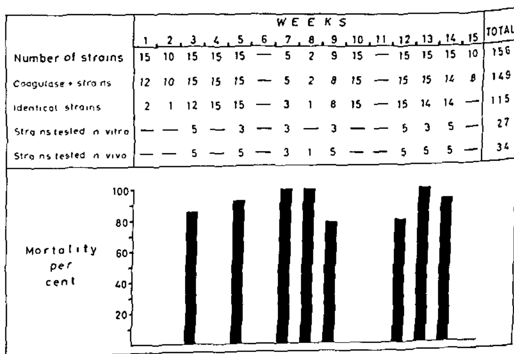
Dust Analysis



	WEEKS 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	TOTAL
Number of strains	15	15	15	15	15	15	15	8	15	15	—	15	—	2	—	160
Coagulase + strains	12	15	15	15	14	7	15	3	11	12	—	10	—	1	—	130
Identical strains	12	15	15	15	14	7	14	2	11	10	—	10	—	1	—	126
Strains tested in vitro	5	—	5	—	—	—	3	—	3	—	—	5	—	—	—	21
Strains tested in vivo	5	—	5	—	3	—	5	—	5	—	—	5	—	—	—	28

Fig 2

Result of mouse pathogenicity tests on strains isolated from dust from the room of the patient with infected eczema The distribution of strains examined by biochemical and identity tests is also shown



	WEEKS 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	TOTAL
Number of strains	15	10	15	15	15	—	5	2	9	15	—	15	15	15	10	156
Coagulase + strains	12	10	15	15	15	—	5	2	8	15	—	15	15	14	8	149
Identical strains	2	1	12	15	15	—	3	1	8	15	—	15	14	14	—	115
Strains tested in vitro	—	—	5	—	3	—	3	—	3	—	—	5	3	5	—	27
Strains tested in vivo	—	—	5	—	5	—	3	1	5	—	—	5	5	5	—	34

Fig 3

Result of mouse pathogenicity tests on strains isolated from dust from the room of the patient with infected burns The distribution of strains examined by *biochemical* and identity tests is also shown

as before by determining the resistance pattern for antibiotics and the diffusible antigens and howed that 295 were identical with infection strains from the respective patients These were distributed thus, case A 126 strains, case B 54 strains, and case C 115 strains The determination of diffusible antigens proved in this connexion to be of great value As a matter of fact the strains could be identified as originating from the respective patients by this method because of differences in the precipitation pattern

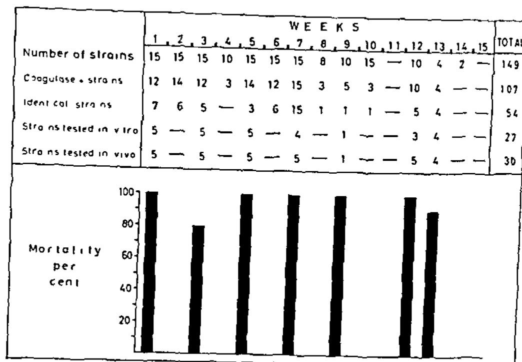
Extensive biochemical analyses was carried out on 75 strains that on the basis of resistance pattern and diffusible antigens had proved to be identical with infection strains This included tests of phosphatase and staphylokinase formation, haemolysin pattern, quantitative determination of alpha lysin and hyaluronidase, and phage typing The results are shown in Table 2

As can be seen all the strains examined were coagulase, phosphatase, and staphylokinase positive All but 2 of the dust borne strains analysed formed alpha lysin only one did not form beta lysin An interesting finding was that 7 dust strains formed beta lysin Quantitative determination of alpha lysins disclosed no great differences The variations were no greater than may be explained by normal errors of the method, however Hyaluronidase formation, which was also measured, showed analogous results Some strains gave higher, and some lower, titres than the original strains

Phage typing All dust strains subjected to detailed biochemical analysis were phage typed with the following results *Case A* All strains but one reacted with phages 6 7/47/53 1034 group III, *viz* the original infection strain The divergent strain reacted with these and in addition with phages 81 and 42E which belong to group I It is noteworthy that all the strains that could be shown to form beta lysin reacted with the same phages as the original strain *Case B* Of the 27 strains isolated from the dust collected in the room of this patient 22 reacted with the same phages as the strain isolated from the burns (5/77/819 1034 group III) The remaining 5, all isolated during the third week of the investigation, reacted with phages 3B 3C/71 group III, and were therefore of another type Note that their resistance pattern for antibiotics was the same as that stated in Table 1 as characteristic of the infection strain *Case C* Like the original, the strains isolated from dust reacted poorl

[illegible] the original strain

Virulence tests embracing 92 strains were carried out on mice The results are shown in Figs 2 4 As can be seen, the virulence of the strains remained unchanged week after week, and no diminution took place The mortality was 100 per cent for strains from Case A, 73 per cent for those from Case B, and 93 per cent for Case C The survivors were killed and examined Abscesses were found in all organs, and



	WEEKS 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	TOTAL
Number of strains	15	15	15	10	15	15	15	8	10	15	—	10	4	2	—	149
Coagulase + strains	12	14	12	3	14	12	15	3	5	3	—	10	4	—	—	107
Identical strains	7	6	5	—	3	6	15	1	1	1	—	5	4	—	—	54
Strains tested in vitro	5	—	5	—	5	—	4	—	1	—	—	3	4	—	—	27
Strains tested in vivo	5	—	5	—	5	—	5	—	1	—	—	5	4	—	—	30

Fig 4

Result of mouse pathogenicity test on strains isolated from dust from the room of the patient with empyema The distribution of strains examined by biochemical and identity tests is also shown

culture resulted throughout in growth of the same strain that had been used to infect the animals The distribution over the 15 weeks of observation of dustborne strains examined by coagulase-, identify- and extensive biochemical tests is also shown in detail in Figs 2–4

DISCUSSION

Experiments were carried out to compare the pathogenicity of staphylococci isolated from clinical infections and the upper respiratory tract with organisms isolated from dust and objects in the patients' environments Three patients were selected who could be assumed to be 'dangerous carriers', and whose environments might be expected to be heavily infected

The three patients fulfilled the criteria of 'dagerous carriers' and investigation of their surroundings disclosed abundant staphylococcus aureus on practically every object examined The strains from the foci of infection showed biochemical similarities, but differed with respect to resistance pattern for antibiotics, phage type, and number of diffusible antigens On the basis of the individual characteristics of the respective strains it was possible to identify isolated environment strains Most of these were identical with the original strain from the focus of infection, and their biochemical properties were unchanged Of 180 strains picked at random 100 fulfilled the requirements for absolute

identity, viz the same biochemical characteristics, the same resistance pattern for antibiotics, and the same diffusible antigens as the strain from the focus of infection Bearing in mind the fact that in making the initial selection regard was paid only to the appearance of the colonies and to pigment-formation, this must be considered a good yield The environment of a dangerous carrier is dominated to a very great extent by the bacteria with which he is infected The patient with the extensive eczema is a good exampel of this no fewer than 52 of the 60 strains initially isolated were identical, using the criteria given, and showed the same biochemical characteristics as the strain from the focus of infection

Comprehensive dust analyses were performed Altogether 465 strains were isolated Of these 386 were coagulase positive, and 295 proved to be identical with the original strains from the focus of infection Determination of the number of diffusible antigens was found to be a particularly useful method, and could be employed to identify the patient from which the dustborne strain originated This was particularly true of strains from Case A, in which the pattern was characterized by a distinct extra line separating it from other staphylococci

Biochemical analyses disclosed that the strains formed, in addition coagulase, phosphatase and stphylokinase With regard to haemolysins, alpha lysin formation could not be demonstrated among a few of the strains but 7 dust strains formed beta lysin, on the other hand, although the infection strain could not be shown to do this Quantitative alpha-lysin and hyaluronidase determinations revealed no reduced activity among the infection strains

Phage typing gave similar results Dust strains reacted with the same phages as the original strain Some strains proved to be of different types however, and it is of interest with regard to these that they showed the same resistance pattern as the original strain Finally, pathogenicity tests on mice largely confirmed what had already been found by other methods The dust strains retained their pathogenicity for mice through out the observation period, no reduction being noted

It may therefore be concluded, to judge from the findings of this investigation that staphylococci do not lose any of their *enzymatic* activity phage typability, or mouse-pathogenicity when they are present in the environment of a patient infected with these organisms or when they are recovered in dried state from dust No difference has been found between strains isolated from infectious *foci and* respiratory passages and most strains isolated from environment and dust No grading of the strains has therefore been possible, and in this respect the results best tally with those of *Thomas et al* (17) It has been shown in previous papers that a large number of the staphylococci present on the skin are characterized by lower activity This discrepancy may be explained by accomodation of the *organisms* to a new environment, with resultant changes

The question of whether the findings of this investigation may permit the drawing of conclusions concerning the pathogenicity of staphylococci in man must remain open It is not yet known what factor with regard to these organisms is of the greatest importance in this respect In the animal experiments we used a method which requires large infection doses The results are not necessarily relevant to natural infection in man Infection trials in man using small infection-doses, as *Elek* (4, 5) has carried out, might possibly better elucidate this point With the methods at present available, however, no difference has been demonstrated with regard to biochemical activity or animal pathogenicity

SUMMARY

Three typical dangerous spreaders of pathogenic staphylococci were selected for this investigation One was suffering from infected generalized eczema, one from extensive infected burns, and one from empyema A large number of strains were isolated from the patients environments and were compared with the original infection strains Several different tests, including enzymatic activity, phage-typability, resistance pattern, and mouse pathogenicity were used No difference could be found between, on the one hand, strains isolated from the focus of infection and respiratory tracts of the patients and, on the other hand, most strains isolated from environment and dust

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SIMULTANEOUS OCCURRENCE OF E. COLI B AND L ANTIGENS IN STRAINS FROM DISEASED SWINE

Influence of Cultivation Temperature
Two New E Coli K Antigens K 87 and K 88

By

IDA ØRSKOV, FRITS ØRSKOV, W J SOJKA and J M LEACH

Received 19 v 61

In recent years there has been a steady increase in the interest in *E coli* strains, especially haemolytic strains isolated from diseases in swine (*Timoney* 1956, *Sojka et al* 1957, *Ewing et al* 1958, *Rees* 1959 *Ørskov et al* 1960)

A number of such strains have been examined at the International Escherichia Centre and some new antigens have been found which will be described in the present paper In the course of these investigations, a number of controversial findings appeared which could only be explained when it was found that a coli strain could contain L and B antigens at the same time Furthermore, it was found by chance that incubation of the culture with an L antigen at a lower temperature than 37° C influenced greatly the agglutinability of this culture These findings seem to have a certain academic interest but are also quite important from the point of view of diagnostic bacteriology

Before presenting the results, the serology of the *E coli* B and L antigens will be reviewed briefly Both are characterized as K antigens As regards their thermostability, they are similar in some respects, i e after heating cultures containing either B or L antigen to 100° C, no K agglutination takes place in sera prepared with live culture (OK serum), neither do such cultures evoke formation of K antibodies The test which is used to distinguish between the two kinds of antigens is the examination of the agglutinin-binding capacity of 100° C cultures A culture containing a B antigen will still be capable of combining with the B agglutinins after heat treatment and the serum will be depleted of these In contrast, the same kind of absorption carried out with an L antigen culture does *not* impair (or impairs only to some extent) the content of L antibodies in the serum Ordinarily live or non-heated cultures of strains possessing either type of K antigen fail to react or react only to a low titre in sera prepared with boiled culture (O sera) until

they have been heated to 100° C Such strains are called O inagglutin able For other properties of the K antigens, the reader is referred to *Kauffmann* (1954)

MATERIALS AND METHODS

St ... the ...

OG 7_1 OG 7_2 the O antigen of 145 will be OG 7_1 G 7 has a strong relationship with 08 and some connection with 032 while 145 has no relationship with 08 but also some relationship with 032 Table 1 gives a survey of the antigens of the five strains G 7 is here assigned to O group 8 while no decision has been taken as regards the O antigen numbering of 145 G 7 has H antigen 19 and 145 antigen 45 The K antigens of the strains will be dealt with later

TABLE 1
Strains Used

Strain No	Antigens			
	O	K		H
		B	L	
RVC 2907	141	85a b→85a c (B)		4
E 68	141	85a b (B)	88 (L)	4
CG 9	141	85a c (B)		*[4]
G 7	8(G 7_1 G 7_2)	87 (B?)	88 (L)	19
145	?(G 7_1)	87 (B?)		45

CG 9 is non motile but motile strains of this serotype have H antigen 4

Preparation of antisera Rabbits were injected intravenously five times at two day intervals with increasing doses ranging from 0 1 to 1 0 ml of the following antigens 18 hours broth cultures *heated to 100° C* for two hours were employed f ... using saline suspensions of 10^9 cells/ml) Bleeding was

... test was performed by suspending plate culture directly in a drop of serum diluted 1 10 Tube agglutinations were carried out as titrations with two fold *serial dilutions* of 1 10 serum and a saline suspension of the culture which was either preserved with 0 5 per cent formalin or heated to 100° C for one hour The tubes with unheated ... 37° C for tw ...

... these mixtures were

... er cent agar added was used for plates

and beef broth for fluid medium In both cases 1 per cent peptone 0 3 per cent NaCl and 0 2 per cent Na_2HPO_4 12 H_2O were added

The plate cultures were incubated at 37° C for 20 hours or at 18° C for two days This prolonged incubation was used in order to obtain a sufficient amount of culture for the experiments

EXPERIMENTAL RESULTS

In Table 2 a survey is given concerning slide agglutination cross tests of the five strains in question It will be seen that all strains are O inagglutinable in the O sera concerned, which means that the agglutination in the OK sera—as H agglutination can be excluded—are conditioned by K antigen reactions It is further evident that RVC 2907 and E 68 are closely related as regards their K antigens and that the same is true for G 7 and strain 145 These relationships are independent of the cultivation temperature Finally a reciprocal K antigen relationship can be found between E 68 and G 7 but only when 37° C cultures are examined, *i e* the K antigen common to E 68 and G 7 is not demonstrable in bacteria grown at 18° C

TABLE 2

Results of Slide Agglutination Tests

Cultures	Cultiv temp	OK Sera RVC2907	E68	G7	145	CG9	* O sera
RVC2907	37° C	+++	+++		–	–	–
	18° C	+++	+++	–			
E68	37° C	++	+++	+++			
	18° C	+++	+++	–			–
G7	37° C		+++	+++	+++	–	
	18° C			+++	+++		–
145	37° C			+++	+++		
	18° C			+++	+++		
CG9	37° C		–			+++	
	18° C					+++	

Serumdilution = 1 10

– Means no agglutination ++ and +++ symbolise different intensities of agglutination

* Each strain was tested in all five O sera corresponding to the strains

The results of cross absorption tests between OK sera of RVC 2907 and E 68 are shown in Table 3 Serum RVC 2907 is depleted of K antibodies by saturating the serum either with homologous heated culture grown at 37° C or with the heterologous heated culture E 68 grown at 37° C This means that both strains are in possession of the B antigen previously numbered K 85 (*Ørskov et al* 1960) It should be added that the titres in OK serum RVC 2907 are rather low New serum preparations did not result in higher titres Similar low-titred sera are seen after immunization with some other coli strains

TABLE 3

Results of Cross Absorption Tests between RVC2907 and F68 (Tube Agglutinations)

Cultures	Cult. temp	RVC2907: O serum unabs	RVC2907: OK serum unabs		RVC2907: OK serum abs by 100° C cult of RVC2907(37°)	RVC2907: OK serum abs by 100° C cult of F68(37°)		F68: O serum unabs	F68: OK serum unabs		F68: OK serum abs by 100° C cult of F68(37°)	F68: OK serum abs by 100° C cult of RVC2907(37°)	
Formalinized													
RVC2907	37° C	20	80		0	0		0	320		0	0	
"	18° C	40	320		0	0		40	640		0	0	
F68	37° C	20	80		0	0		20	1280		640	1280	
"	18° C	40	160		0	0		40	640		0	0	
Heated to 100° C		nc	c	nc	nc	c	nc	nc	c	nc	nc	c	nc
RVC2907	37° C	640	2560	320	0	0	0	320	2560	320	0	0	0
"	18° C	1280	5120	1280	80	160	80	1280	5120	2560	320	320	0
F68	37° C	320	2560	320	0	0	0	320	1280	160	0	0	0
"	18° C	320	2560	320	20	0	20	640	5120	640	0	0	0
		1	2		3	4		5	6		7	8	

Figures in brackets indicate cultivation temperature
Titre is given as reciprocal value of the highest serum dilution displaying a macroscopical reaction in tube titration
0 means negative in dilution 1 : 20
c means that the culture was washed in saline and resuspended in this medium to original density
nc means that the heated cultures were used directly
Serum RVC2907 contained H agglutinins and before absorptions were carried out the serum was depleted of these; this did not alter the agglutination results recorded here
The results are arranged for convenience in columns numbered at the bottom

According to the definition of L antigens, the absorption of serum E 68 by homologous heated culture cultivated at 37° C reveals that L *antibodies are left in the serum reacting with the E 68 culture grown* at 37° C, while no agglutination of the 18° C culture takes place When the absorption of serum E 68 is carried out with culture RVC 2907, antibodies are likewise still present which are capable of combining with the 37° C culture of E 68 The O inagglutinability of an 18° C culture of E 68 in homologous O serum (see Table 3, column 5 and Table 5, column 1) implies the presence of a K antigen under these growing conditions also *As the 18° C culture of E 68 fails to agglutinate in the* homologous OK serum absorbed by heated 37° C culture, the culture seems to lack the L antigen, and the above-mentioned inagglutinability must be ascribed to the B antigen The L antigen found in strain E 68 has received the new K antigen number K 88

Turning to the results obtained with heated cultures recorded in Table 3, it will be seen that the O titres are rather low This could be caused by a low content of antibody in the sera, but under conditions not recorded here high O titres were found in the same sera Most probably the low O titres are caused by heat stability of the K antigen greater than that usually found in B antigens It will be noted that *washing of cultures used for agglutination increases the titres considerably* Heating to 120° C did not give unequivocal results

In all four absorbed sera (Table 3, columns 3, 4, 7 and 8) some agglutinins are left which react with heated culture of RVC 2907 grown at 18° C As titrations with the three other heated cultures showed depletion of the cross absorbed sera, RVC 2907 and E 68 can be considered identical as regards their O antigen It will be seen from the biochemical patterns recorded in Table 4 that RVC 2907 and E 68 belong to the same biotype and, as mentioned above, both strains have H antigen 4 These two strains represent L and L+ forms of the same sero-fermentative type —

RVC 2907 = 0141 K 85 (B) H 4
E 68 = 0141 K 85 (B), K 88 (L) H 4

At this juncture it would be appropriate to mention strain CG 9, which probably should be described as a variant of this same serofermentative type (for fermentation reactions, see Table 4) This 0141 strain was received from *Kelen* and co-workers and has already been described by them (1959)

Kelen et al, who used E 68 as a reference strain, found that E 68 had an L antigen and that CG 9 had a B antigen No relationship was found between these K antigens Examination in this laboratory showed that *CG 9 had* a B antigen different from that of RVC 2907, even though some relationship was found between the two B antigens Strains similar to CG 9 were also received from laboratories in England where this type has been referred to as E 145 (*Sojka et al* 1960) Among English

strains was one received from *Leach*, Newmarket (Bayer Ltd). This latter strain, 232 19, was isolated from a swine oedema autopsy from heart blood in mixed culture with a strain of serotype 0141 · K 85 (B) · H 4 As both strains gave the same biochemical pattern and had the same O and H antigens, some sort of variation between the two K antigens was sought When single colonies from platings of the 0141: K 85 strain were examined, one out of 40 colonies gave a typical agglutination in the OK serum produced with CG 9 (dilution 1: 10), while the remaining 39 colonies agglutinated in K 85 serum only (dilution 1 10) When new platings were made from K 85 reacting colonies, they again split off colonies reacting only in CG 9 OK serum with a frequency of 1 30 to 1 40 In a series of consecutive platings of colonies reacting in K 85 serum, it was invariably found that a few colonies contained the K antigen of CG 9 A number of stock cultures with the serotype 0141 K 85 H 4 showed the same phenomenon It has not yet been possible to find the reverse variation, *i e* to isolate colonies equipped with K 85 antigen in a CG 9 culture The finding, which is only briefly mentioned here, is probably the effect of a sort of variation comparable to a certain extent with the O form variation in *Salmonella* (*Kauffmann* 1940) This is the first case of this type of variation described in the E coli group A more detailed report is in progress and will be published later

The practical question remains—should the K antigen of CG 9 be labelled with a new K antigen number This is not a question of academic interest only, as strains of this serotype are frequently found in oedema disease in swine In Canada, for example, the majority of haemolytic coli strains isolated from cases of oedema disease belong to this type

In cross absorption experiments using OK sera of RVC 2907 *and* CG 9 it was found that, apart from a weakly developed common K antigen factor, they both had special K factors It has been decided to place the K antigen of CG 9 as a K subtype of K 85 and the following serotype formulas are therefore proposed for the two types in question.—

RVC 2907 = 0141 K 85a, b→85a, c (B), H 4
CG 9 = 0141 K 85a, c (B) H—

where "a" symbolises the weak common K factor and → symbolises the variation from the K 85b factor to the K 85c factor

Some doubt exists as to the fine structure of the O antigen of CG 9, as small amounts of agglutinins for the homologous heated culture will be left when O serum CG 9 and O serum 141 (RVC 2907) have been cross absorbed It cannot be decided whether these weak agglutinations are caused by some B agglutinins in the O sera or by special O factors in the strains

Similarly some doubt remains in connection with the common K

factor "a" Future investigations might show that this factor could be assigned to the common O antigen (0141) Serological and biochemical analyses of the relationship between O and B antigens are in progress in different laboratories and it might be that the distinction between O and B antigens will be difficult to draw very sharply

TABLE 4

Biochemical Reactions of the Strains Examined

	BVC2907	E 68	CG9	G7	H5
Adonitol	–	–	–	–	–
Dulcitol	+	+	+	+	+
Sorbitol	+	+	+	+	+
Arabinose	+	+	+	+	+
Xylose	+	+	+	+	+
Rhamnose	+	+	+	+	+
Maltose	+	+	+	+	+
Salicin	$+^{2}$	$+^{3}$	$+^{4}$	–	–
Inositol	–	–			–
Lactose	+	+	+	+	+
Sucrose	–	–	–	+	+
Mannitol	++	++	++	++	++
Glucose	++	++	++	++	++
Sorbose	–	–	–	+	+
Indole	+	+	+	+	+
Gelatin		–			–
Ammonium glucose	+	+	+	+	+
Ammonium citrate	–				–
KNO_3	+	+	+	+	+
Voges Proskauer	–	–	–	–	–
Methyl red	+	+	+	+	+
Urea	*	*	*	–	–
KCN test		–			–

* Occasionally strains of these serotypes are positive in the urea test

It should be stressed here that the practical diagnosis of these two 0141 types necessitates the use of two OK sera corresponding to the two types, as the common factor "a" will not manifest itself in slide agglutination using diluted sera

Only a limited number of strains have been tested for the K 85a, b–85a, c variation and, until it has been shown that this variation is a universal phenomenon for all strains carrying the K 85b antigen, the K designation K 85a, b (B) will be used for such strains Therefore the serotype of E 68 will be E 68 = 0141 K 85a, b (B), K 88 (L) H 4

Finally it could be mentioned that some preliminary experiments show that strains with K antigen K 85a, c (E 145) are more toxic for mice than are strains with K 85a, b (E 68)

The interrelation between E 68 and G 7 is recorded in Table 5 After absorption of OK serum E 68 with homologous heated culture grown at 37° C, agglutinins were still present for the homologous E 68 and for the heterologous G 7 culture cultivated at 37° C (see column 3 and compare with column 7 in Table 3) This indicates that the cross re-

TABLE 5

Results of Cross Absorption Tests between I 68 and G7 (Tube Agglutinations)

Cultures	Cultiv temp	I 68: O serum	I 68: Ok sera	I 68: Ok sera absorbed by				G7: O serum	G7: Ok sera	G7: Ok sera absorbed by			
		unabs	unabs	I 68 (37°) 100°	G7 (37°) live	1) I 68 (37°) 100° 2) G7 (18) live	1) I 68 (37°) 100° 2) G7 (37°) live	unabs	unabs	G7 (37°) 100°	I 68 (37°) live	1) G7 (37°) 100° 2) I 68 (18°) live	1) G7 (37°) 100° 2) I 68 (37°) live
Formalinized													
I 68	37° C	40	2560	1280	160	640	0	0	2560	1280	0	1280	0
„	18° C	80	640	0	320	0	0	0	0	0	0	0	0
G7	37° C	20	2560	2560	0	2560	0	0	2560	2560	320	2560	160
„	18° C	0	0	0	0	0	0	0	2560	320	640	320	320
Heated to 100° C													
I 68	37° C	320	1280	0	–		–	0	0	0	–	–	–
„	18° C	1280	5120	0			–	0	0	0	–	–	–
G7	37° C	0	20	80	–	–		5120	2560	0	–	–	–
„	18° C	0	0	0		–	–	5120	5120	640	–	–	–
		1	2	3	4	5	6	7	8	9	10	11	12

– Means not carried out
In columns 5, 6, 11 and 12 0 means negative in serum dilution 1 : 40, as the first tube was omitted because of small amounts of absorbed sera
In addition, see key to Table 3

action between the two strains can be ascribed to the L antigen By means of additional absorption of the serum with live culture of G 7 grown at 37° C (column 6) the L agglutinins were removed In contrast, an 18° C culture was not capable of removing these (column 5) Removal of antibodies from serum E 68 exclusively be means of 37° C live culture of G 7 caused depletion of the serum for L antibodies combining with G 7, while the titre of E 68 grown at 37° C was only lowered, and that of the 18° C culture was unimpaired (column 4) This result supported the conception that the reaction of E 68 grown at 37° C in homologous OK serum includes both an L and a B agglutination

The conclusion to be drawn at present is that E 68 and G 7 carry the same L antigen = K 88 Just as E 68 in addition possesses another kind of K antigen, this also seems to be true as regards G 7 (see right half of Table 5), but the antigenic structure is not as intelligible as that of E 68

Apart from the content of agglutinins for the L antigen common to G 7 and E 68, the "pure" L serum of G 7 (Table 5, column 9) exhibits a peculiar trait, namely the content of antibodies which react with both non-heated and heated homologous culture when grown at 18° C Successive absorption of the "pure" L serum by live 37° C culture of E 68 did not eliminate the reaction of G 7 regardless of cultivation temperature (Table 5, column 12) Had the examination of the 18° C cultures been omitted, the results would imply the existence of an L antigen or an L antigen factor at G 7 different from that common to E 68

In order to throw some additional light on the K antigen of G 7, a more detailed examination of the relationship between G 7 and 145 was carried out This latter strain has no antigenic relationship with E 68 It is evident from Table 6 that both non-heated and heated 145 cultures react in OK serum G 7 but not in O serum G 7 In contrast, nonheated and heated cultures of G 7 agglutinate both in OK and O serum 145 In both cases the cross reactions are independent of cultivation temperature Saturation of OK serum G 7 and 145 with a boiled culture of 145 removes all agglutinins in OK serum 145 In OK serum G 7 there are still agglutinins left for non-heated G 7 culture when grown at 37° C, and for both 37° and 18° C heated G 7 culture (column 5) In O serum 145, all cultures are partly O-inagglutinable, thus demonstrating the presence of a K antigen According to the outcome of absorption tests, strain 145 should have a K antigen of the B type One of the strange things is that this strain is capable of removing the agglutinins for nonheated 18° C G 7 culture from OK serum G 7, *i e* a K agglutinin, while the homologous strain grown at 37° C is not This paradoxical result led to an attempt to absorb OK serum G 7 with a boiled culture of the homologous strain cultivated at 18° C (Table 6, column 6) By this procedure depletion of the serum was obtained for all agglutinins except the L agglutinins, and furthermore the same culture could remove both O and K agglutinins from OK serum 145

TABLE 6

Results of Cross Absorption Tests between G7 and 145 (Tube Agglutinations)

Cultures	Cult's temp	G7						145				
		O serum		OK serum				O serum	OK serum			
		unabs	abs by 100° cult of G7(37°)	unabs	abs by 100° cult of			unabs	unabs	abs by 100° cult of		
					G7(37°)	145(37°)	G7(18°)			145(37°)	G7(37°)	G7(18°)
Formalinized												
G7	37° C	0	0	1280	640	640	640	160	640	0	80	0
"	18° C	0	0	1280	160	0	0	160	640	0	640	0
145	37° C	0	0	1280	160	0	0	320	1280	0	640	0
	18° C	0	0	640	160	0	0	80	640	0	640	0
Heated to 100° C												
G7	37° C	2560	0	1280	0	1280	0	2560	640	40	0	0
	18° C	2560	0	2560	1280	1280	0	5120	5120	20	1280	0
145	37° C	0	0	2560	2560	0	0	5120	5120	0	5120	0
	18° C	0	0	2560	2560	0	0	5120	5120	0	2560	0
		1	2	3	4	5	6	7	8	9	10	11

See key to Table 3

(Table 6, column 11) A new O serum of G 7 was prepared in the usual way, *i e* by immunization with a boiled culture grown at 37° C This time the titre could not be recorded as zero, but as twenty when 145 was tested after treatment with formalin or heat However, when the O serum was *prepared with a boiled culture of G 7 cultivated at 18° C* antibodies were present which reacted with unheated culture to a low titre but with heated 145 culture to a high titre (Table 7) In this connection it should be noted that heated G 7 culture grown at 37° C (Table 6, column 2) was capable of depleting the 37° C O serum but not the OK serum of antibodies reacting with heated culture grown at 18° C (column 4) A similar absorption of the 18° C O serum was not undertaken All the results obtained point to the fact that both the agglutinin-binding capacity and the agglutinogenic capacity were better maintained in the heated culture of G 7 grown at 18° C than in that grown at 37° C Whether these properties refer to the O antigen or to an OK antigen complex cannot be stated

TABLE 7
Comparison between Different G7 O Sera (Tube Agglutinations)

Cultures	Cultiv temp	O sera G7		
		(37°) old	(37°) new	(18°)
Formalinized				
G7	37° C	0	40	80
	18° C	0	80	160
145	37° C	0	20	80
	18° C	0	20	80
08	37° C	0	20	0
032	37° C	–	20	20
Heated to 100° C				
G7	37° C	5120	5120	2560
	18° C	2560	5120	2560
145	37° C	0	20	2560
	18° C	0	20	2560
08	37° C	1280	5120	2560
032	37° C		0	640

See key to Table 3
– Means not carried out

The antigenic structures of G 7 and 145 have not been completely clarified as yet, it can only be stated that the results indicate that G 7 and 145 have the same K antigen In G 7 grown at 37° C this K antigen seems *to behave in the same way* as a thermolabile L antigen, but in G 7 grown at 18° C and in 145 independent of cultivation temperature it behaves in the same way as a B antigen In addition, G 7 and 145 are related as to their O antigens

TABLE 8

Results of Cross Absorption Tests between O Sera of G7 and 145

Cultures heated to 100° C	Cult. temp	O serum G7 (18°)		O serum 145 (37°)	
		unabs	abs by 100° cult of 145 (37°)	unabs	abs by 100° cult of G7 (18°)
G7	37° C	5120	5120	640	0
"	18° C	5120	5120	2560	0
145	37° C	2560	0	2560	0

See key to Table 3

It is evident from Table 8 that a boiled culture of G 7 grown at 18° C is capable of depleting O serum 145, while no decrease in the homologous titre is obtained by absorption of the 18° C G 7 O serum by culture 145 This means that G 7 has the complete O antigen of 145, and in addition a strong special factor When these results are compared with the results mentioned previously regarding different absorptions of the OK sera, the facts can be defined as follows —

Live culture G 7, 37° C, produces O antibodies $G\,7_1$ $G\,7_2$
100° C culture G 7, 37° C, produces O antibodies $G\,7_2$
100° C culture G 7, 18° C, produces O antibodies $G\,7_1$ $G\,7_2$
Live or 100° C culture 145, 37° or 18° C,
produces O antibodies $G\,7_1$

In the seroformulas given below, G 7 has been assigned to O group 8, even though the O antigen is not identical with test type 08 The O antigen of 145, having no relation to test type 08, is called 0? Both strains agglutinate to some extent in O serum 32, and test strain 032 reacts in O serum 145 and in O serum G 7, but only when this is produced with the 18° C culture In other words the mode of reaction is similar to strain 145, but in contrast to test culture 08, which also agglutinates in the 37° C O serum of G 7 (see Table 7)

As it is an open question whether the K antigen common to G 7 and 145 can be described as a B *or an L antigen*, this antigen will be numbered K 87 (B?) According to this, the seroformulas of G 7 and 145 will be —

G 7 = 08 K 87 (B?), K 88 (L) H 19
145 = 0? K 87 (B?) H 45

The strange K antigen of G 7 has at least no direct connection with the L antigen common with E 68, but it might be supposed that the mere presence of this L antigen in the 37° C culture influenced the condition of the other K antigen in some way It is *hard to believe* that this is so for the following reason —From G 7, it is possible to isolate on a bromthymol blue lactose medium colonies with an appearance

deviating from the usual. These colonies have lost the E 68 G 7 L antigen. A non-heated culture from such an L^- colony behaves in slide and tube agglutination in the same way as G 7 grown at 18° C. An OK serum produced with the 18° C culture contained L antibodies reacting with E 68 grown at 37° C culture. This shows that the L antigen was not completely suppressed in the 18° C culture used for immunization. This might be an incorrect statement, as it cannot be excluded that L antigen was formed after the culture was injected into the animal. The experiment must be repeated with a culture treated with formalin instead of a live one. The L^- culture of G 7 does not cause formation of L agglutinins reacting with E 68, thus pointing to the complete lack of the L antigen, and yet this serum also contained agglutinins which could not be removed by absorption either with homologous heated L^+ or L^- culture grown at 37° C.

It was stated above that strain G 7 splits off colonies lacking the E 68/G 7 (= K 88) L antigen. On bromthymol blue-lactose plates these colonies differ in appearance from the L^+ colonies, while they are the same on the broth-agar medium used throughout this study. It might then be inferred that the 18° C culture of G 7 possibly originated from such L^- colonies, or from a mixture of the two variants in which the L^+ variant had gradually disappeared during sub culture. However, this was not the case. Even after several sub-cultures at 18° C, the agglutinability in OK serum E 68 was fully restored in new sub-cultures cultivated at 37° C for 24 hours. The development of the L antigen was suppressed just as suddenly when the incubation temperature was changed the other way round from 37° to 18° C. E 68 behaved in these respects in the same manner. It can be mentioned here that the cultures were more opaque, and especially E 68 more mucoid, when grown at 18° than at 37° C, perhaps indicating a better development of an M antigen at this temperature.

Further evidence in favour of the possibility of the simultaneous existence of B and L antigens was produced by means of a genetic experiment. Strain G 7 happens to be a genetic donor strain, which means that it carries a fertility factor (F) which it is able to transmit to other coli strains. Genetical experiments have not been undertaken on a large scale, but it is sufficient to state here that another coli strain with a B antigen can in addition acquire the L antigen common to G 7 and E 68 when it is converted to the donor state through contact with G 7.

As mentioned above, the simultaneous presence in the same strain of L and B antigens cannot be detected by the conventional method for K antigen determination. The difference in the development of an L antigen at different cultivation temperatures offered a means—at least in the present case—of differentiating between L and B antigens. For obvious reasons it was decided therefore to screen the existing L antigen test strains for additional B antigens. The agglutinability of non-heated culture grown at 37° and at 18° C was examined in O sera, and

in OK sera unabsorbed and absorbed by homologous culture heated to 100° C No B antigens were detected in the 18° C cultures, *i e* no case was observed where, in contrast to the 37° C culture, an O inagglutinable 18° C culture failed to react in the absorbed serum In many cases the 18° C cultures were fully agglutinable in the corresponding O sera, and in agreement with this fact no or only a very faint reaction was seen in the absorbed sera, thus showing that the formation of the L antigen in these cultures has been suppressed In other cases both cultures were inagglutinable in O serum and both reacted in the absorbed serum Seven B antigen test strains were examined as regards their O inagglutinability In six cases the 18° and 37° C cultures failed to react in the O sera In the remaining case the 18° C culture was O agglutinable while the 37° C was not

DISCUSSION

The present study covers several aspects of coli serology, and in addition the labelling of some new K antigens One aspects is the suppression of the L antigen at 18° C, another the introduction of strains furnished with two types of K antigens Yet another is the appearance of a K antigen which in one strain presents itself as a B antigen while in another the same antigen can hardly be characterized as being either L or B or something else, as the properties of the antigen vary at different temperatures Another aspects which perhaps is worth notice is that all the strains involved are isolated from pigs

As regards the first point, the suppression of an antigen at 18° C, similar experiences have been described previously *Rowland* (1914) showed by means of wet film Indian ink preparations that B *pestis* was clothed with an envelope when propagated at 37° C, while the culture grown at 20° C developed no envelopes Later *Schütze* (1932) found this envelope associated with an antigen which was more readily produced at 37° C than at 26° C *Felix, Bhatnagar & Pitt* (1934) described how the Vi antigen of B typhosus (*S typhi*) strains was suppressed at cultivation temperatures between 20° and 25° C, and also between 40° and 44 5° C At these temperatures the resistance to O agglutinins was annulled *Jude & Nicolle* (1952) and *Nicolle & Jude* (1952) made a more extensive study into the influence of cultivation temperature on the development of serologically identical Vi antigens On the basis of Vi agglutinability, O inagglutinability, capacity to absorb Vi agglutinins and sensibility to Vi bacteriophages they were obt[illegible]

[illegible] All investigators find the inhibition of the Vi antigen entirely reversible

[illegible]

gen, designated L antigen The Vi and L *antigens resemble each other* in many ways but differ in others The main difference between the two antigens is that the binding capacity of the L antigen is destroyed by *heating for 1–2* hours at 100° C No report has appeared regarding the influence of the cultivation temperature on the amount of L antigen in *E coli*, but *Kurimoto, Suzuki & Fuju* (1954) described some findings along these lines in an *Alcalescens-Dispar* strain The strain was claimed to possess a K antigen This was because of lack of antisera only recognized by its O *inagglutinability As Frantzen (1951) has demonstrated* that several of the K antigens of *Alcalescens-Dispar* strains are serologically identical with L antigens of *E coli*, it may be assumed that the O inagglutinability described by *Kurimoto et al* was due to the presence of an antigen of the L type Those authors reported that the K antigen was formed at 28° but not at 24° C They also found that incubation on media containing more than 0 5 per cent glucose inhibited K antigen formation In this laboratory this latter fact could not be *confirmed as regards the G 7/E 68 L antigen (= K 88) described here*

Furthermore the present paper reports that a screening of several strains with serologically different L antigens did not show suppression of the L antigen at 18° C in all cases It was at first thought that this might imply that possibly more kinds of so-called L antigens were involved On the other hand, it must be taken into consideration that even serologically identical Vi antigens exhibit different behaviour in different strains

The whole problem of suppression of antigens bears some resemblance to data obtained in quite another field of microbiology *Sparck* (1956) found that two *Neurospora crassa* cultures form strain specific spore antigens when grown at 25° C, while a different but strain common antigen is found at 35° C As no absorptions could be carried out with the technique employed, *Sparck* could not tell to what extent this difference was qualitative From our own experiments with *E coli* it has been found that temperature influenced the amount of L antigen That *it was probably not a complete inhibition* was best seen from the fact that some agglutinogenic power of the L antigen was maintained in the 18° C culture This pointed more to a quantitative than to a qualitative difference at the two cultivation temperatures

The main question which arises concerning the action of cultivation temperature on the antigen formation is the cause of this effect In *Paramecium aurelia* where, in contrast to *E coli* (as far as is known) there is a mutual exclusion of different antigens, *Sonneborn* & *Beale* (see *Beale* 1952) demonstrated that the temperature determines which one of a series of alternative antigens is being formed It was shown by crossing experiments that different genes at independent loci are concerned with *antigen production at different temperatures* The state of the cytoplasma suppresses or allows the action of the genes Whether a similar mechanism is involved in *E coli* is unknown but it would

certainly be worth while using a combined genetical and immunological approach to the problem of antigenic variation

Usually the suppression of the L antigen formation in *E coli* grown at 18° C will mainly be of academic importance, as coli strains generally only have one type of K antigen However, when in addition strains possess another type, the problem is also important as regards practical serological typing When it is stated that coli strains with both L and B antigens have not been found hitherto, it should be added that this is perhaps because such strains have not been looked for That they have been disclosed now was only because of the occurrence of cross reacting strains *Knipschildt* (1945) points to the possibility of B and L antigens being present at the same time but considers this to be unlikely He adds that in practice it will be impossible to demonstrate B antigens in strains provided with L antigens, as the L titre will mask the presence of a B titre This is also true if the two antigens are equally well formed under all possible environmental conditions In the present work it was reported that almost all the B antigen test strains examined were O inagglutinable also when grown at 18° C, and that many L antigen test types became O agglutinable at that temperature It was inferred therefore, that at least these last mentioned strains were only provided with L antigens Other procedures may be applied in order to examine the possibility of a more complex antigenic structure in strains forming L antigen at 18° C

The phenomenon of the existence of strains with two kinds of K antigens is reminiscent of a finding reported previously (*Ørskov & Ør*

tic

st

into any of the known *E coli* antigen groups On account of the heat lability it had been justifiable to say that the antigen was more comparable to an L antigen than to any of the other antigens F conversion experiments indicated that the f antigen was carried over from donor to recipient strain It is reported in this study that in a similar conversion experiment also the L antigen G 7/E 68 (= K 88) can be transferred from G 7 to a strain having a B antigen It is considered that this speaks in favour of comparing the K 88 L antigen with the f+ antigen

The third problem mentioned in the introduction to the discussion was the finding of a K antigen, the properties of which varied dependent on the strain, and within one of the strains dependent on the cultivation temperature This problem has not been investigated sufficiently at the present stage, but suffice it to say that it might be due to the O-B complex lacking certain properties in strain G 7 when cultivated at 37 C *Kauffmann & Vahlne* (1945) and *Vahlne* (1945) called ...

... *Vahlne* also states that after heating to

100° C, L antigens can combine with L agglutinins to some extent, so that low L titres may be eliminated by absorption This shows the difficulty which sometimes exists in differentiating between L and B antigens Genetical experiments which are in progress confirm that L and B antigens are different, *i e* that they are determined by different genetical factors, but in practice they can be hard to distinguish from each other

In fact, a thorough reinvestigation of the nature of the B antigens is called for *Knipschildt* (1945) demonstrated three different B antigens *in a rather large* material and consequently the B antigen was thought to be a rare type of K antigen Subsequent studies have shown that B antigens are very common, and are perhaps the most frequent among the *E coli* K antigens (see also *Ørskov* 1956) *Knipschildt* demonstrated the B antibodies by absorption of an OK serum by a boiled culture of a strain from the same O group containing an L antigen or a heterologous B antigen Since then many B antigens have been described, but only in few cases has it been possible to use this approach because strains with identical O antigens but with different B or L antigens are not easily found Another possibility for demonstration of B antibodies would be absorption of an OK serum with a K variant of the homologous strain While this procedure has been feasible when working with L and A antigens, it has always failed when attempts were made to produce pure B sera Therefore, in most cases—and also in the present paper—the demonstration of B antigens has been based on the inagglutinability of live, or at least non-heated culture, in O sera and the heat stability of the agglutinin-binding capacity of the strains

All the principal strains dealt with in this paper were isolated from pigs When it is taken into consideration that the same antigens were found in different combinations in the strains, one is inclined to think that special conditions either in the animals themselves or in the piggery favour the exchange of genetic material between the strains

SUMMARY

The present paper describes the serological analysis of a number of interrelated *E coli* types frequently isolated from oedema disease and enteritis in swine

(1) Strains with both B and L antigens are described

(2) The L antigen is established as a new K antigen, numbered K 88 (L)

(3) Development of this L antigen is suppressed at 18° C cultivation temperature

(4) Several, but not all, known L antigens will show this temperature dependence

(5) In one strain the *K 88 (L) antigen is present together* with the already known K antigen 85 (B)

(6) In another strain possessing the K 88 (L) antigen, an unknown K antigen (probably of the B type) is found at the same time. This is numbered K 87 (B?).

(7) Strains exist which possess K 85 (B) or K 87 (B?) without the K 88 (L) antigen.

(8) The detection of the B antigen is not influenced by cultivation temperature.

(9) The strain carrying the K 87 (B?) and K 88 (L) antigens is a genetical donor strain. The L antigen can be transferred from this strain to other coli strains along with a fertility factor.

(10) Preliminary details are given regarding a new type of antigenic variation in *E. coli* K antigens. In certain cultures a constant low percentage of cells is found which have a B antigen different from that of the parent organism, i.e. either one or the other B antigen is expressed. This variation occurs in strains having the originally described K 85 (B) and is symbolised as follows: K 85a b→a c (B). The a factor is a weak factor common to the parent and the variant cultures.

(11) In one strain the properties of the O (or O B) antigen is partly damaged at 100° C when the culture has been cultivated at 37° C but not when grown at 18° C. In other words, the boiled 18° C culture behaves in the same way as a normal boiled culture, whereas the boiled 37° C culture does not.

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AUS STATENS SERUMINSTITUT KOPENHAGEN (INTERNATIONALE SALMONELLA UND ESCHERICHIA CENTRALE) UND DEM VETERINÄR-BAKTERIOLOGISCHEN INSTITUT DER UNIVERSITÄT BERN

EINE NEUE SALMONELLA-SPECIES: SALMONELLA LOCARNO = 57:z_{9}:z_{12}

Von

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Eingegangen 7. XI. 61

Eine neue *Salmonella species* wurde im Veterinär Bakteriologischen Institut der Universität Bern aus einem grossen Gürtelschweif (*Cordylus giganteus*) isoliert und mit *Salmonella locarno* bezeichnet.

Das Tier wurde 1960 aus Südafrika importiert und mit 50 anderen Tieren derselben [illegible] zu Futterverweigerung.
Aus dem Abszesseiter wurden *Aeromonas* und *Proteus* isoliert, aus Herz und Leber *Aeromonas* und nach Tetrathionat Anreicherung *Salmonella* Bakterien, die auch aus dem Darm gezüchtet wurden.

Die neue *Salmonella-species* verhielt sich in der biochemischen Untersuchung wie folgt: keine Spaltung von Adonit, Lactose, Saccharose und Salicin, keine Indol-Bildung und keine Spaltung von Urea. Gelatine (nach *Kohn-Fautrop*) wurde nach 2–3 Tagen verflüssigt. In Ferrochlorid-Gelatine wurde H_2S prompt gebildet, während die Gelatine nach einigen Wochen verflüssigt war (der genaue Zeitpunkt dieser Verflüssigung wurde nicht festgestellt). Prompte Spaltung von Arabinose, Dulcit, Glukose (mit Gas), Inosit, Maltose, Mannit (mit Gas), Rhamnose, Sorbit, Trehalose und Xylose.

Es sei bei dieser Gelegenheit nachgetragen, dass die von uns früher beschriebene *Salmonella-species S. bern* = 1,40:z_4,z_{32}:– Maltose und Mannit (mit Gas) prompt spaltete.

S. locarno ergab eine positive Reaktion in Stern's Glycerinfuchsin-Bouillon nach 1 Tage und positive Reaktionen in flüssigen Ammonium-Media mit Glukose und Natrium-Citrat. Nitrat wurde reduziert, die *Voges-Proskauer* Reaktion war negativ und die Methylrot Reaktion positiv. Von den organischen Säuren waren d-Tartrat und Natrium-Citrat nach 2 Tagen positiv, während l- und i-Weinsäure nach 14 Tagen negativ waren. Mukat war nach 1 Tage und Natrium-Malonat nach 2 Tagen positiv. Der KCN-Test war negativ.

Auf Grund der Gelatine Verflüssigung der prompten Dulcit Spaltung und des Verhaltens in organischen Säuren kann diese neue *Salmonella species* zum *sub genus* II gerechnet werden

Die serologische Untersuchung ergab, dass die neue *Salmonella species* die Formel 57 : z_{29} : z_{14} besitzt, also ein neues *Salmonella* O Antigen enthält, das mit dem *Arizona* O Antigen 34 auf Grund gekreuzter Absorptions Versuche identisch ist

ZUSAMMENFASSUNG

Es wird eine neue *Salmonella species* *Salmonella locarno* = 57 : z_{29} : z_{14} aus einem verendeten grossen Gürtelschweif isoliert beschrieben Gleichzeitig wird hierdurch eine neue *Salmonella* O Gruppe 57 aufgestellt

LITERATUR

F Kauffmann H Ley & I Steel Eine neue *Salmonella species Salmonella bern* = 1 40 z_4, z_{23} Acta path et microbiol scandinav *50* 335 1960

PASTEURELLA HAEMOLYTICA VAR. UREAE

Action on Blood Agar and Serological Reactions

By

S. D. HENRIKSEN

Received 7.vi.61

Since the last report (5) the number of strains of Pasteurella haemolytica var. ureae isolated in this institute has increased to 9 from 8 different patients, 8 isolated from the nose and one from sputum. So far only one strain, a strain from a maxillary sinus, has been shown to be the cause of disease. The others were all present in fairly large numbers in the cultures, but their role as pathogens is uncertain. It is the purpose of this paper to report some new observations concerning the action on blood agar of these strains, and also some serological studies.

ACTION ON BLOOD AGAR

In previous papers (3, 4, 5) it was stated that our strains regularly produce a green discoloration with a more or less marked, but usually faint partial haemolysis. Throughout the period of time when these studied were carried out, our blood agar was prepared from a commercial dehydrated blood agar base, stated to give a pH of 7.4, by addition of human bank blood containing a citrate anticoagulant solution. In this same period we had frequently been dissatisfied with the performance of our blood agar, mainly because β haemolytic streptococci tended to give green zones with little genuine haemolysis. Various experiments led to the conclusion that the main reason for this was that the blood [illegible] is was due to the tap water or

[illegible] base before addition of blood was adjusted to pH 0.8, more satisfactory results were generall[illegible] tained [illegible]
the gree[illegible]
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In order to confirm th[illegible]

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Auf Grund der Gelatine-Verflüssigung, der prompten Dulcit-Spaltung und des Verhaltens in organischen Säuren kann diese neue *Salmonella-species* zum *sub-genus* II gerechnet werden.

Die serologische Untersuchung ergab, dass die neue *Salmonella-species* die Formel 57: z_{29}: z_{42} besitzt, also ein neues *Salmonella* O-Antigen enthält, das mit dem *Arizona* O Antigen 34 auf Grund gekreuzter Absorptions-Versuche identisch ist.

ZUSAMMENFASSUNG

Es wird eine neue *Salmonella-species: Salmonella locarno* = 57: z_{29}: z_{42}, aus einem verendeten, grossen Gürtelschweif isoliert, beschrieben. Gleichzeitig wird hierdurch eine neue *Salmonella* O-Gruppe 57 aufgestellt.

LITERATUR

F. Kauffmann, H. Fey & F. Steck. „Eine neue *Salmonella-species: Salmonella bern* = 1,40: z_4 z_{32}: –" Acta path. et microbiol. scandinav. *50*: 335, 1960.

Agglutination tests were set up by mixing twofold serum dilutions starting at 1 10 with equal volumes of saline suspensions of living organisms Agglutination
slow and
, likewise
with the

The capsular reaction tests were carried out by mixing a loopful of twofold serum dilutions starting with undiluted serum with a loopful of very thin bacterial suspension The reactions were read by phase contrast microscopy

The complement fixation tests were carried out by a conventional technique using 2 100 per cent haemolytic units of complement and incubation times of 1 hour for fixation and 10 minutes for haemolysis

RESULTS

Results of agglutination tests are shown in Table 1 Our strains fall into two groups with minor mutual cross reactions The 4 strains of P haemolytica var haemolytica likewise fall into two groups, one consisting of two strains obtained from the National Collection of Type Cultures, London, and the second of two Norwegian strains from the Veterinary Institute, Oslo Cross-reactions were weak and of low titer

TABLE 1

Agglutination Reactions of Strains of Pasteurella haemolytica var ureae and var haemolytica in Corresponding Immune Sera

Antigen strains	Immune sera against strain			
	585 61	218 60	T 20	9712
Var ureae				
3520 51	1280	80	<20	<20
218 60	80	2560	<20	<20
3369 60	160	1280	<20	<20
4027 60*	1280	80	<20	<20
585 61	1280	160	<20	<20
KS 1427 61	1280	320	<20	<20
4334 61	80	2560	<20	<20
1805 61	160	2560	<20	<20
Var haemolytica				
T 20	20	<20	1280	10
T 185	20	<20	1280	10
9712	80	40	<20	1280
9280	40	20	<20	1280

* Strain 3974 60 isolated from the same patient gave the same reactions as this strain

The Complement fixation tests, shown in Table 2 gave similar results Cross reactions were very slight or none The strains of P haemolytica var ureae when tested with the homologous type serum, showed distinct prozone phenomena in low serum dilutions up to 1 24 or 1 48

One of the serological types of P haemolytica var ureae gave positive capsular reactions in the corresponding serum (anti 585 61) in serum

NaOH-solution The two portions were used to prepare blood agar by addition of identical quantities (8 per cent v/v) of the same sample of human blood The two portions were prepared simultaneously and under identical conditions After the blood agar plates made from these two portions of agar had solidified, two plates of each portion were inoculated with each of our strains, and one of the plates was incubated in a closed jar in a humid athmosphere, the other in an ordinary incubator at 35° C The plates were checked after 24 and 48 hours

In order to check the pH of the two sets of plates, the agar was removed from one plate of each portion, crushed, put in a centrifuge tube and centrifuged until a small quantity of fluid had been pressed out The pH of this fluid was determined by means of indicator paper strips and was found to be between 6 7 and 6 8 in the unadjusted portion and 7 2 in the portion which had been adjusted to pH 8 with alkali

On the more acid plates all strains of P haemolytica var ureae produced distinct green discoloration with more or less confluent growth Of 4 strains of P haemolytica var haemolytica 3 (9380, T20 and T185) produced a greenish discoloration with little or no sign of haemolysis, whereas the fourth (9712) produced distinct although weak zones of β-haemolysis

On the more alkaline plates none of the strains produced green discoloration, the strains of P haemolytica var ureae produced no haemolysis, but all 4 strains of var haemolytica produced distinct zones of β-haemolysis

Thus the results indicate that P haemolytica var ureae only produces the characteristic green discoloration and partial haemolysis when the blood agar is on the acid side of the neutral point, and is entirely inactive on the alkaline side

Furthermore, the β-haemolysis produced by some strains (3 out of 4) of P haemolytica var haemolytica is dependent upon the pH and tends to disappear on the acid side of the neutral point It was surprising that such small changes could have such a marked effect

The results make it clear that P haemolytica var ureae in agreement with previous suggestions is not truly haemolytic, and the question then arises, whether it might not be better to consider this organisms as a separate species rather than a variety of P haemolytica However, the two organisms show so many other similarities that it is felt that such a change may not be required for the time being, but should be left until our state of knowledge about these organisms has advanced further

SEROLOGY

Methods

Rabbit immune sera were produced by intravenous injections of saline suspensions of living organisms from blood agar cultures with intervals of 4 to 5 days A considerable number of injections had to be given and the dosage had become very high before satisfactory sera were obtained

suggests the possibility that these sera may have contained incomplete antibody In this connection it may be recalled that live antigen heavy dosage and prolonged immunization appear to favor development of incomplete antibodies against other organisms (2)

SUMMARY

Pasteurella haemolytica var ureae produces a distinctive green discoloration and weak partial haemolysis on blood agar on the acid side of neutrality (pH 6 7 6 8) but is entirely inactive on the alkaline side (pH 7 2)

Some strains of P haemolytica var haemolytica produce β haemolysis at a slightly alkaline reaction (pH 7 2) but not at a slightly acid reaction (pH 6 7 6 8)

P haemolytica var ureae is not a truly haemolytic organisms and the question whether it should be considered as a separate species is discussed It is suggested that this decision should be left for the future

P haemolytica var ureae can be divided into two serological types one of which (type 1) has a type specific capsular antigen

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dilutions up to 1 · 8, whereas all other strains gave negative reactions in their homologous type sera. The strains which reacted, all grew with distinctly more mucoid and slimy colonies than the others.

TABLE 2
Complement Fixation Reactions of Strains of Pasteurella haemolytica var. ureae and var. haemolytica in Corresponding Immune Sera

Antigen strains	Immune sera against strain	
	585/61	218/60
Var. ureae		
3520/59	6144	<24
218/60	<24	6144
3369/60	24	6144
4027/60	3072	<24
585/61	3072	<24
KA 1427/61	3072	<24
4334/61	<24	6144
1805/61	<24	6144
Var. haemolytica		
T 20	<24	<24
T 185	<24	<24
9712	<24	<24
9380	<24	<24

* Pasteurella haemolytica var. ureae gave marked serum against the homologous sero type in low

Thus it is clear that P. haemolytica var. ureae can be divided into at least two serotypes. The fact that 8 strains of quite different origin, geographically and epidemiologically, could be referred to one or the other of two types, suggests that the number of types in Norway may be moderate. One of the two types, which it is reasonable to designate type 1, since it contains the type strain (3520/59), has a type specific capsular antigen (and probably a specific somatic antigen as well), whereas no capsular antigen was demonstrated in the second type, type 2.

It is reasonable to ascribe the strong and compact agglutination of type 1 strains in dilutions up to 1 : 20 as well as the capsular reactions, to capsular antibody, whereas the finely granular and very slow reactions in higher dilutions of this serum, and of the other type in the homologous serum probably is due to a different kind of antibody.

None of the two types showed significant cross reactions with any of the two types of P. haemolytica var. haemolytica, but since this organism can be divided into at least 11 types (1), this result is of limited significance.

The tendency of our two types to show prozone phenomena both in agglutination and complement fixation with the homologous type sera,

suggests the possibility that the ... antibody In this connection ... dosage and prolonged immun... incomplete antibodies against ...

Pasteurella haemolytica ... coloration and weak parti... of neutrality (pH 6 7–6 8), ... (pH 7 2)

Some strains of P haemoly... at a slightly alkaline reaction ... action (pH 6 7–6 8)

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1 *Biberstein F L Gills M & ...* lytica Cornell Vet., 54 ...
2 *Glenchur H Zinneman H ...* body in experimental ...
3 *Henriksen S D & Jyssum K* the human respirat... 1960
4 *Henriksen S D & Jyssum K* human respiratory t...
5 *Omland T & Henriksen S* ureae isolated from ... 53 117 1961

...re from recurrent herpes ... e all isolated and passaged ... gens

...h 0 5 ml of undiluted virus ... r complete degeneration of ... with the cells were frozen ... ubes at —60° C A fresh tube ... used without centrifugation ... pared similarly except that

...m were immunized 4 times ... as indicated in Table 1 A ... l schedule with the control

...g Titers of Pooled Sera ... Cell Culture Antigen

...mplex CF antibody titer	
...	Immunized with control cell culture antigen
	<4
	<4
	<4
	<4

...tigens but in ... the cells had ... urpose the ... were stored

... 2 full ... units ... nits in ...

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585/61	3072	<24
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4334/61	<24	6144
1805/61	<24	6144
Var. haemolytica		
T 20	<24	<24
T 185	<24	<24
9712	<24	<24
9380	<24	<24

Antigens prepared from strains of Pasteurella haemolytica var. ureae gave marked prozone phenomena with immune serum against the homologous sero type in low dilutions (up to 1 : 24 or 1 : 48).

Thus it is clear that P. haemolytica var. ureae can be divided into at least two serotypes. The fact that 8 strains of quite different origin, geographically and epidemiologically, could be referred to one or the other of two types, suggests that the number of types in Norway may be moderate. One of the two types, which it is reasonable to designate type 1, since it contains the type strain (3520/59), has a type specific capsular antigen (and probably a specific somatic antigen as well), whereas no capsular antigen was demonstrated in the second type, type 2.

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The tendency of our two types to show prozone phenomena both in agglutination and complement fixation with the homologous type sera,

Each of the 12 herpes simplex virus isolates tested were from recurrent herpes simplex vesicles on skin or mucous membranes. They were all isolated and passaged 1–4 times in HeLa cells before the preparation of CF antigens.

Immunization Antigens

Guinea pig kidney cell cultures were inoculated with 0.5 ml of undiluted virus. The bottles were incubated at 36.5° C. Immediately after complete degeneration of the cells had taken place, usually in 2 days, the fluids with the cells were frozen and thawed, harvested and stored in rubber stoppered tubes at —60° C. A fresh tube was taken for each immunization and the material was used without centrifugation.

A control cell culture immunization antigen was prepared similarly, except that no virus was inoculated.

Immunization of Animals

A group of 5 young guinea pigs weighing about 200 gm were immunized 4 times with intraperitoneal injections of the virus antigen as indicated in Table 1. A similar group of animals were immunized by an identical schedule with the control cells culture antigen.

TABLE 1

The Immunization Schedules and herpes simplex CF Antibody Titers of Pooled Sera of 5 Guinea Pigs Immunized with the Virus or Control Cell Culture Antigen

Time in weeks after the first immunization	Successive immunizations	Herpes simplex CF antibody titer	
		Immunized with virus	Immunized with control cell culture antigen
0	1.0 ml intraperitoneally	< 4	< 4
4	1.0 ml intraperitoneally		
8	1.0 ml intraperitoneally		
9		256	< 4
24	2.0 ml intraperitoneally	64	< 4
26		128	< 4

Complement fixing Antigens

CF antigens were prepared in the same way as the immunization antigens but in HeLa cells. The fluids were harvested immediately after all or nearly all the cells had degenerated but were still adherent to the glass surface, for which purpose the cultures were examined microscopically twice a day. The CF antigens were stored at —25° C.

Complement fixation Test

CF tests were made by *Bengtson*'s technique with [illegible] units of complement being used [illegible] were used in identification tests [illegible] the antibody titration. A titratic [illegible] included in each test. The titer [illegible] unit was usually 1:16 but varied in a few tests by one tube up or down. A control titration of complement in the presence of each tested antigen was also included in each test.

RESULTS

Herpes simplex Guinea Pig Immune Serum

The herpes simplex CF antibody responses of guinea pigs immunized with guinea pig kidney cell culture grown virus are shown in Table 1. One week after the third immunization with 1.0 ml of virus the CF

FROM THE DEPARTMENT OF VIROLOGY, UNIVERSITY OF HELSINKI, FINLAND

IDENTIFICATION OF HERPES SIMPLEX VIRUS ISOLATES BY A COMPLEMENT FIXATION TECHNIQUE

By

PEKKA HALONEN and SIMO TARPILA

Received 14.xi.61

The more extensively cell culture methods are routinely used in virus isolations, especially from throat specimens, the more of a problem does the identification of herpes simplex virus isolates become.

In many cell culture systems the cytopathogenic effect of herpes simplex virus isolates may be difficult to distinguish from that of some enteroviruses. For the identification and typing of the latter, a complement fixation (CF) technique has been reported by a number of investigators (3, 5, 6, 7, 8, 9, 11). Therefore it was felt desirable to develop a similar identification procedure for herpes simplex virus isolates.

This report describes the preparation of herpes simplex immune serum suitable for CF studies and the methods used in the successful identification of 12 herpes simplex virus isolates in HeLa cells by this technique.

MATERIALS AND METHODS

Cell Culture

HeLa cells were grown in small Carrel flasks in a medium containing 40 per cent human serum in Hanks' solution. At the time of virus inoculation the cultures were washed three times with Hanks' solution and 7 ml of a maintenance medium containing 5 per cent horse serum, 5 per cent tryptose phosphate broth and 90 per cent Eagle's minimum essential medium (2) was added to each flask. The number of cells per flask was at that time approximately 6 million.

Guinea pig kidney cell cultures ... on
methods from the kidneys of 2 5 da...
10 per cent calf serum and 0.05 per...
and the maintenance medium 10 per...
bumin hydrolysate in Earle's solution.

Virus Strains

The herpes simplex virus strain "A H" used in the preparation of the immunization antigen was isolated in 1959 in HeLa cells from a typical herpetic vesicle and identified by a neutralization test. It had been passaged twice in HeLa cells and twice in guinea pig kidney cell cultures.

This study was supported by grants from the *Sigrid Juselius Foundation* and the *Finnish State Council for Natural Science*.

The technical assistance of Mrs *Hilkka Ala-Vainikola* and Miss *Sirkka Kerminen* is gratefully acknowledged.

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0	1 0 ml intraperitoneally	< 4	< 4
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antibody titer of the pooled serum of 5 guinea pigs was 1 256 A fourth immunization with 2 0 ml of virus after a 15 week rest period did not increase the titer *No CF activity with herpes simplex CF antigen* was obtained in the pooled serum of 5 guinea pigs immunized with a control cell culture antigen

The specificity of herpes simplex *immune serum taken in the final* bleeding was tested with poliomyelitis type 1 and adenovirus type 1 CF antigens prepared identically with the herpes simplex CF antigen No reaction with these antigens could be noted at a serum dilution of 1 4

Identification of herpes simplex Virus Isolates

Each of the herpes simplex virus isolates tested gave a complete 4 + fixation with 8 antibody units of guinea pig immune serum The CF antigen titers were between 1 2 and 1 16 There was no correlation between the number of passages of the virus isolates and the antigen titer No antigens were significantly anticomplementary In addition to the standard maintenance medium used in the study, various other modifications were tested, *but they* all gave similar results One such test is shown in Table 2

In addition to HeLa cells primary trypsinized human embryonic *fibroblasts and human amnion cells of a continuous line, strain Utrecht*, obtained from Dr *R Doorschodt*, Hygienisch Laboratorium der Rijks Universiteit, Utrecht, Holland, were tested for the preparation of CF antigens It was found, however, that all herpes simplex virus isolates, whether adapted to HeLa cells or on primary passage, multiplied very poorly in the amnion cells and no virus CF activity was observed in these harvests In fibroblast cultures the CF antigen titers were significantly lower than in HeLa cells

TABLE 2

The Reactions of herpes simplex CF Antigens Prepared in Various Media with herpes simplex Guinea Pig Immune Serum and Control Cell Culture Guinea Pig Immune Serum

Medium used in the preparation of CI antigen	Reaction with herpes simplex immune serum						Reaction with control cell culture immune serum
	Antigen dilutions						Antigen dilution
	1 1	1 2	1 4	1 8	1 1ι	1 32	1 1
Eagle's m * + 5% h s § + 5% trypt †	4	4	4	4	1	0	0
Eagle s m * + 5% h s §	4	4	4	4	1	0	0
Eagle s m * + 10% h s § + 5% trypt †	4	4	4	4	2	0	0
Eagle's m * + 10% h s §	4	4	4	3	0	0	0
Medium No 199 + 10% h s §	4	4	4	4	1	0	0

* Tagle s minimum essential medium § Horse serum † Tryptose phosphate broth
4 = no hemolysis, 3 2 1 = different degrees of hemolysis 0 = complete hemolysis

DISCUSSION

The identification procedure generally used for herpes simplex virus isolates is the neutralization test on chorioallantoic membranes of chick embryos, the pock count being used as indicator (8). Our experience, however, has been that the herpes simplex virus strains isolated in cell cultures do not always produce clearly demonstrable pocks on chorio allantoic membranes in primary passage. On the other hand, in the herpes simplex neutralization test in cell culture tubes difficulties have been encountered with the "break through" phenomenon. In such a test even with high concentrations of hyperimmune herpes simplex anti-serum isolated foci of degenerated cells begin to appear in the cell culture tubes and slowly increase in size. The interpretation of such an identification test is difficult.

According to the results of the present study, the CF typing test could be used as an additional idenfication procedure for herpes simplex virus isolates in laboratories where CF tests are in routine use.

Preparation of a potent virus CF antigen from herpes simplex isolates should not be difficult if a suitable cell culture system is selected. In the experiments reported, the CF antigen titers in HeLa cells were usually 1:4 or 1:8. A potent herpes simplex CF antigen can also be prepared in chick embryo tissue culture as reported by *Schmidt, Lennette & Sohn* (10) and rabbit kidney cell culture as reported by *Artzet* (1).

In the preparation of a suitable immune serum for the herpes simplex CF test the earlier experiments were made with chick embryo grown virus but the virus CF antibody titers in guinea pigs were low (from 1:4 to 1:16). Purification of the cell culture grown virus with fluoro carbon (4) for the preparation of herpes simplex immunization antigen could not be done because the virus antigenicity was completely removed before the host antigenicity. The preparation of the herpes simplex immunization antigen in a strictly homologous cell culture system with guinea pig kidney cells and guinea pig serum produced in 9 weeks an immune serum of high CF antibody titer (1:256) and with no CF activity against the control antigens of HeLa cell cultures.

Since this study was made, several herpes simplex virus strains received from the State Serum Institute, Helsinki, by the courtesy of Dr. K. *Penttinen* have been identified by the CF technique.

SUMMARY

The identification of 12 herpes simplex virus isolates in HeLa cells by a complement fixation technique is reported.

HeLa cells, primary trypsinized human embryonic fibroblasts and human amnion cells of a continuous line were tested for the preparation of CF antigens. In HeLa cells the antigen titers were highest and no significant anticomplementary effect was noted.

A herpes simplex immune serum with a high CF antibody titer and no activity against host antigens of HeLa cell cultures was prepared in guinea pigs by immunizing the animals with guinea pig kidney cell culture grown virus

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TRANSACTIONS
OF THE SWEDISH PATHOLOGICAL SOCIETY

Meeting December 3, 1960

G Moberger DIAGNOSIS OF THYROID CARCINOMA HISTOLOGICAL CLASSIFICATION

The following modified classification has been followed at the Institute of radio pathology since 1960

TABLE 1

Histological Classification of Thyroid Carcinoma

GRADING

I *Differentiated cancer*
- 1 Highly differentiated
  - A Predominantly or purely papillary
  - B Mixed papillary and follicular
  - C Predominantly or purely follicular
- 2 Moderately differentiated
  Partly solid with preserved follicular structures
- 3 Low differentiated
  Predominantly solid with identifiable follicular structures
- 4 Hurthle-cell carcinomas
- 5 Epidermoid carcinomas

II *Undifferentiated cancer*
- 6 Small cell carcinomas sarcoma like
- 7 Giant cell carcinomas

STAGING

- I Solitary encapsulated tumors with invasive growth only in the capsule or the closest adjacent thyroid tissue
- II Encapsulated multiple tumors and tumors invading the major part of the thyroid gland
- III Tumors growing outside the thyroid gland and cases with metastases

The experience so far has showed a good correlation between the various types of tumors and their biological behaviour such as the degree of clinical malignancy, the radio iodine uptake and the radiosensitivity Thus the highly differentiated follicular types of carcinomas have a greater tendency to accumulate radio iodine

than the lower differentiated tumors The undifferentiated carcinomas of the small cell type *are usually highly radiosensitive* but also many of the low differentiated more or less solid tumors may be considerably radiosensitive

S *Franzén* and J *Zajick* DIAGNOSIS OF THYROID CARCINOMA ASPIRATION BIOPSY OF THE THYROID GLAND

During the last five years the authors investigated the *usefulness of the* aspiration biopsy in the diagnosis of tumors of the thyroid gland The aspiration biopsies were performed with a thin needle (22 gauge) connected with a Luer lock syringe having a special handle which *permits a singlehanded operation* (*Franzén s syringe* KIFA Solna 3 Sweden) The use of a thin needle and the short aspiration time employed (1 2 seconds) minimized the admixture of blood

The aspiration biopsy of thyroid tumors was performed *in more than 400 cases* From the results so far obtained it can be concluded that the application of this method in most cases gives valuable diagnostic informations

1 *By the inside palpation of the tumor with the needle soft solid fibrotic* stony hard—calcified vascular, pulsative etc

2 By the macroscopic examination of the material obtained solid tissue particles *old blood pus serous fluid* etc

3 By the cytologic analysis of the smear preparations The difficulty in distinguishing the highly differentiated carcinomas from benign epithelial hyperplasia has to be pointed out *On the other hand* the highly differentiated thyroid carcinomas can readily be recognized in the metastatic growth

J *Einhorn* DIAGNOSIS OF THYROID CARCINOMA CLINICAL CONSIDERATIONS

Only one half of patients surgically treated for thyroid carcinoma have correct *preoperative diagnoses In many cases of slow growing thyroid carcinoma there are* no clinical signs that might facilitate differential diagnosis between cancer and benign nodular goiter

Thyroid carcinoma is usually suspected on the basis of anamnestic data or findings at palpation Scintigraphy is one of the preoperative aids to diagnosis It may help us to establish the diagnosis of thyroid cancer if uptake of radioiodine can be demonstrated *in the metastases Although uptake of radioiodine is often* demonstrable in a thyroid carcinoma it is generally less than that in normal thyroid tissue A defect in the scintigraphic record of the thyroid gland will therefore be suggestive of malignancy

Multiple microscopic neoplastic lesions are demonstrable in approximately one half of patients with papillary thyroid carcinoma frequently even in the grossly *healthy l be The clinical significance of these microscopic lesions however* is not yet fully elucidated

G *Östberg* GRANULOMA MALIGNUM

A 40 year old woman had ulceration of the nasal cavity and lesions on the outer surface of the nose Biopsy showed polymorphous necrotizing granulation tissue without any definite tumourous character Treatment with cortisone and roentgen produced temporary improvement The patient died 11 months after onset and then had a large local recurrence

Post mortem examination revealed pronounced swelling of the face *thickened* mucosae of the nasal sinuses and enlarged firm lymph nodes of the neck The liver

and kidneys showed multiple pea sized yellow-white patches Large tumour like foci were also seen in the kidneys These lesions, like some microscopic foci in the lungs and bronchi, showed very polymorphous tissue with a pronounced tendency to necrosis In addition to inflammatory cells numerous polymorphous reticulum cells were found with a number of plump giant cells The histological picture resembled that seen in lymphogranulomatosis maligna Hodgkin while the clinical picture was not suggestive of this disease The case illustrates the difficulties encountered in the diagnosis of 'malignant granulomas of the nose'

H Henrikson PELIOSIS HEPATIS

Recently peliosis hepatis was seen as a by finding in 2 cases examined at the department of Pathology, Malmo In these 2 cases the patients were elderly women with advanced mammary cancer They had been treated for a long time with sex hormones, oestrogenic and androgenic derivatives Despite widespread metastases the liver showed no signs of invasion In addition to typical peliosis foci they showed marked stasis with partial destruction of the parenchym in some areas

In recent years some cases of peliosis have been published in which the patients have been treated with hormones An interesting possibility—though difficult to judge—in the present cases is that the hormone therapy might have paved the way for peliosis hepatis

A Lindgren MILIARY CHOLANGIOMATOSIS WITH DEVELOPMENT OF CANCER

G F Voigt FAT EMBOLISM AND PNEUMONIA

During the last 2 years we have had the opportunity of examining 33 patients who had survived traumatic injury for at least 7 hours These cases may be divided into the following groups according to the sites of lesions

1 Mainly skull and brain injuries (13)
2 Mainly thoracic injuries (13)
3 Injuries of the limbs only (6)

In all cases in *groups 1 and 2* inflammatory changes could be demonstrated histologically in the lungs (irrespective of the presence of any fat embolism) The pneumonia was probably due to the cerebral or thoracic *injuries In group 3* focal pneumonia could be demonstrated in only one case the cause of death was cerebral fat embolism It would thus seem that pulmonary fat embolism can hardly be a common cause of fatal pneumonia This is supported by the *results of experimental* studies intravenous injection of homologous fat never produced pneumonia in rabbits that were allowed to live up to 14 days after the injection

F Bergman EXPERIMENTAL CRYPTOCOCCOSIS

The course of infection after subcutaneous injection of *Cryptococcus neoformans* in mice was followed histologically and bacteriologically A large number of animals died from disseminated *cryptococcosis* with widespread inflammatory changes particularly in the lungs Animals that died late in the course [illegible]

[illegible] different phases with mycetemia could be distinguished The first phase occurred during the first few hours after the injection,

the second in association with a generalization of the cryptococcal infection 2 16 days after the injection and the third phase in association with grave cryptococcal lesions in the central nervous system The histological changes in the organs closely resembled those described in human beings

F Bergman & F Linell CRYPTOCOCCOSIS AS A CAUSE OF PROTEINOSIS ALVEOLARIS PULMONUM

Lung changes with the character of proteinosis alveolaris were described in 1953 by *Linell Magnusson & Norden* in a case of cryptococcosis (Acta Dermato Venereologica 33, 1953) The lesions which *in all respects resembled those described in* proteinosis alveolaris pulmonum, were conceived as secondary to cryptococcosis In an investigation of experimental cryptococcosis in mice by *Bergman* it was *found that on regression of the lung changes the alveoli in* some areas were *filled* with large macrophages with PAS positive granules in the cytoplasm The cells closely resembled the so called septal cells in proteinosis alveolaris pulmonum in man *The findings thus suggest that* proteinosis alveolaris pulmonum *might sometimes* be caused by cryptococcal infection

G Hultquist U-B Sundqvist J Thorell STUDIES OF THE α CELLS IN THE *ISLETS OF LANGERHANS*

According to quantitative investigations *of the* pancreatic islets in guinea pigs after repeated subcutaneous injections of cobalt salts the change in the α cells is likely to be of progressive nature However the cause of the characteristic vacuolisation of the α cells it not known After injection of cobalt salts hyperglycaemia is observed in guinea pigs but the blood sugar changes can probably not be responsible for the α cell changes Such changes in the α cells can rather be seen after hypoglycaemia The cobalt induced hyperglycaemia is somewhat reduced in guinea pigs treated with hexametonium before the cobalt injection No effect of hexametonium on the cobalt induced vacuolisation of the α cells is noted however In adrenalectomised guinea pigs on the other hand neither hyperglycaemia nor vacuolisation of the α cells does appear after injection of cobalt salts

Adrenaline (1 5 mg/kg b w) was administered in subcutaneous injections to guinea pigs during 4–5 days and the effect of the injections on the pancreatic islets was studied by means of karyometry An increase of the nuclear size of the α cells was noted *but* no vacuolisation of the cytoplasm *The increase of the size of the* α cell nuclei was not more than about 8 % in comparison to about 25 % *after cobalt* treatment Judging from those results the adrenaline administration seems to have a stimulative effect on the α cells The cobalt effect however probably is not merely due to an action of adrenaline released from the adrenal medulla *but* is of a more complicated nature

H Knutson PANCREATIC ISLET CYTOLOGY IN RATS ON A HIGH FAT DIET

A karyometric study of the pancreatic islet cells was carried out in adult male Wistar rats on a slightly hypolipotropic *high* fat diet Nuclei were *measured in* 7 μ sections stained by Gomori s chromalum hematoxylin method using a direct micro planimetric procedure After 3 and 6 weeks the animals showed a statistically significant decrease in α cell nuclear size *(8 % numerical reduction in mean nuclear* area) The β cell nuclear size also decreased (4 1 % after 6 weeks)

The results were discussed with regard to a hypothetic α cell functional activity in fat metabolism, but the α cell nuclear change could possibly also be related to a slight hyperglycemic tendency in rats on the high fat diet However, the concomitant reduction in β-cell nuclear size then seemed paradoxical It was concluded that endocrine activity as revealed by pancreatic islet cell morphology must be complex and not easily elucidated in long term experiments of this kind

L Lidberg EXPERIMENTS ON SENECIOSIS

The hepatotoxic alkaloids monocrotaline and retrorsine, both isolated from various Senecio and Crotalaria plants, were administered subcutaneously to pregnant and non pregnant female rats

The livers of the non pregnant rats showed centrilobular, haemorrhagic necrosis, proliferate endophlebitis and after a few weeks severe fibrosis, megalocytosis and bile duct proliferation

The pregnant rats were highly resistant towards these alkaloids Even at doses high above thosen given to the non pregnant rats no changes were seen In the livers of the embryos one could observe slight centrilobular necrosis, the alkaloids were thus able to pass the placenta

The early toxic effects were studied in the newborn rats by means of intravital staining with trypanblue It was shown that both the nuclei of the liver cells and of the venous endothelial cells were simultaneously stained blue

A Brun & N Jonsson DEMYELINATION IN MALIGNANT LYMPHOMA

Report of a case of generalized reticulum cell sarcoma the initial symptom of which was impairment of hearing progressing to complete deafness

Post mortem examination revealed generalized reticulum-cell sarcoma with diffuse invasion of the soft meninges and of some of the Virchow Robin spaces

The pons showed a discrete unspecific diffuse demyelination of the pyramidal pathways The transverse pathways of the pons showed a bilateral partial and in some places symmetric, demyelination with abundant fatty granular cells and moderate gliosis without atypia or mitoses No cystic softenings perivascular myelin destructions haemorrhages or thrombi Tumour growth was seen surrounding and invading some parts of the acoust [illegible]

Etiologic possibilities were d [illegible]

One might consider the possibi [illegible]

by *Åström et al* (Brain *81* 93 1958)

Demyelination processes of different morphology and pathogenesis are evidently capable of occuring in malignant lymphoma

I Brunk HISTOLOGICAL INVESTIGATION OF PHOSPHATASES IN THALLIUM POISONED GOLDEN HAMSTERS

Changes in the alkaline phosphatase content could be demonstrated in the kidneys small intestine testes *and adrenals In the* kidneys there was a great loss of the enzyme The decrease was most marked in animals killed one week after receiving a dose that normally killed the animals in 2 weeks *About 8* hours after a single moderate dose the thin band of alkaline phosphatase immediately above the nuclei disappears from the small intestine

The amount of alkaline phosphatase in the testes decreases in proportion to the dose and time the poison is allowed to act

The hamster females have a small amount of alkaline phosphatase in the adrenal cortex This amount increases substantially 5 6 hours after administration of a sublethal dose The males have a considerably larger amount of the enzyme which also appears to increase somewhat in thallium poisoning

The content of acid phosphatase decreased in the kidneys and in the small intestine In the kidneys the decrease was proportional to the dose and the period the animals were allowed to survive In the small intestine of poisoned animals only a thin layer of the enzyme was demonstrable immediately above the nuclei between the nuclei and the cuticular border

G Sköld EFFECT OF CADMIUM POISONING ON TESTES

In guinea pigs and rabbits no pathologic changes could be demonstrated in the testes despite repeated injections of 1 mg of cadmium acetate/100 g bodyweight In hamsters the metal produced the same type of changes as those known to occur in rats and mice In addition the testis capillaries were liner by a homogeneous eosinophilic substance which in some sections contained collections of somewhat deformed nuclei while in other sections no nuclei were demonstrable Suspecting that the injurious effect of the cadmium on the testes might be due to its migration into the vascular walls specimens were studied by the sulphidesilver method Within one hour and a half of the injection small black granules could be demonstrated in the endothelial cells of the capillaries of the testes The results suggest that the toxic effect of cadmium is due to its migration into the vessels of the testes

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Meeting April 15, 1961

N O Berg ON THE EFFECT OF CONTRAST MEDIA ON THE RABBIT KIDNEY

S Orell LOCAL TISSUE CHANGES AFTER VACCINATION AGAINST INFLUENZA

G Voigt HISTOCHEMICAL INVESTIGATIONS ON ZINC

N Ehrenberg A CASE OF ANGIOMATOUS LIVER CIRRHOSIS

N Forsby ADENOMATOID DYSPLASIA OF THE LUNG

U Bruhl & G Sköld OSSIFICATION OF THE PHALANGES IN FOETUS

N Stormby THE EFFECT OF SALICYLATE AND CORTISONE ON HETEROLOGOUS TRANSPLANTATION OF CANCER

C G Ahlström N Forsby & N Jonsson ROUS SARCOMA IN RATS AND HAMSTERS

Rous chicken sarcoma (strain Mill Hill) transferred subcutaneously into new born rats grew for a short while in some of the animals and then regressed

On the other hand inoculation of new born rats with cellular and cell free material of another Rous chicken sarcoma (Schmidt Ruppin strain) was followed within 4 weeks by the appearance of lymphogenous cysts and progressively growing

sarcomas in about three fourths of the animals The rat sarcomas could not only be transplanted in series in rats but also re transferred to chickens

The same chicken sarcoma material induced polymorphocellular sarcomas in new born and adult hamsters within 14 days 3 months The sarcoma could be serially transmitted in hamsters and re transferred to chickens

Chromosome analysis of the rat sarcomas showed a chromosal pattern of rat type No precipitation lines were found in the gel between rat sarcoma extract and anti chicken sarcoma sera

N *Jonsson* ROUS SARCOMA IN MICE

Attempts to transfer Rous sarcoma (Strain Mill Hill) to mice with cellular and cell free material have been mainly unsuccessful as in the earlier investigation

After subcutaneous or intramuscular injection of cell-containing material from Schmidt Ruppin strain of Rous sarcoma into new born up to four days old Swiss mice progressively growing tumours developed locally in 18 out of 84 animals after 17 114 days The histological picture was that of a polymorphocellular sarcoma partially a spindle cell sarcoma Metastases in the lungs were seen in three animals Hemorrhagic lesions were not observed The tumours have successfully been transplanted to full grown mice and carried through seven passages They have been re transferred to hens Attempts to transfer the tumour with cell free material have hitherto been unsuccessful

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A. Ehrenberg: A CASE OF ANGIOMATOUS LIVER CIRRHOSIS

A. Forsby: ADENOMATOID DYSPLASIA OF THE LUNG

U. Brunk & G. Sköld: OSSIFICATION OF THE PHALANGES IN FOETUS

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TRANSACTIONS OF THE MEDICAL MICROBIOLOGY DIVISION OF THE SWEDISH MEDICAL SOCIETY

Meeting December 2–3, 1960

Gard S, Böttiger, M & Lagercrantz, R Institute of Virology, Karolinska Institutet, Stockholm LIVE POLIOVIRUS VACCINE

Small scale tests with the CHAT type 1 strain have been conducted since the winter 1957/58

Occurrence of contact infections of susceptible family members was dependent on the age of the vaccinees In families where the virus was fed to a child less than 2 years old spread occurred in 45 per cent If the index child was more than 2 years old contact infections were observed in only 4 per cent The limited spreading capacity of the CHAT strain was confirmed in another study where only 3 out of 119 susceptible contacts became infected after feeding of the virus to 10 per cent of the pupils in a school for blind children

A variation to d+ from the original d character was quite frequent (about 40 per cent) On a few occasions (approximately 5 per cent) a moderate gain in intrathalamic neurovirulence in monkeys was observed

The following immunization schedule was suggested Primary immunization with inactivated vaccine at the age of 6 to 12 months possibly in combination with TPD vaccine Provided that all members of the household have had inactivated vaccine live virus of type 1 is given at the age of 2 years Types 2 and 3 are not at present under consideration

Arro L, Böttiger M, Fagraeus A, Heller L, Kallings L O, Melen B, Olin G, Salenstedt R & Wendel R The State Bacteriological Laboratory, Stockholm
SWEDISH EXPERIENCES OF VACCINATION USING KILLED POLIOMYELITIS VACCINE

The Swedish experiences of polio vaccination are presented (a) in the form of the result of studies of the antibody formation in children with no antibodies against the three types of polio virus prior to vaccination ("triple negative") and (b) as tabulations of the morbidity rate of paralytic polio in vaccinated and unvaccinated humans

A gradual improvement in the activity of the vaccines was noted during 1957–1959 In 1959 an antibody response to type 1 was obtained in 90 6 per cent of the children after two vaccine injections and to type 2 or 3 in 99 4–99 7 per cent After three injections the antibody response was 100 per cent to all types

The protective action as judged by the morbidity rate of paralytic poliomyelitis in vaccinated and unvaccinated individuals of the same age group and during the same period of time has been estimated at about 83 per cent following two injections and about 96 per cent after three injections of vaccine

Discussion

Snedmyr A Serological results for instance so called conversion rates may not be strictly comparable if the methods used for antibody demonstration vary May the fact that the results of the Swedish vaccinations appeared less satisfactory during 1957 than later on be due at least partially to the shift in 1958 to a more sensitive technique for demonstration of neutralizing antibody?

The decrease in the number of paralytic cases during the last few years as compared with earlier interepidemic periods is probably not as marked as shown by the figures since cases not caused by poliovirus have only recently been eliminated Thus the incidence of paralytic RSSE now estimated at about 25–50 cases a year may have been as high earlier although the cases were then diagnosed as poliomyelitis

Norlin G *Salk* (J A M A 169 1829 1959) detected antibodies with one preparation in 99 96 100 00 and 99 96 per cent for the three types in 4716 vaccinated Per cent figures entirely depend on technique Biological assay without standard is not reliable

The results of poliomyelitis vaccination are still open to discussen (Brownlee J Am Statist A 50 1005 1955 Knowelden Brit Med J *5122* 620 1959)

Norden A Dep of Medicine *Lund University* *STUDIES OF THE INACTIVATION OF HEMOLYTIC COMPLEMENT BY HUMAN GAMMA GLOBULIN—ANTI HUMAN GAMMA GLOBULIN*

The quantitative precipitin curve between comercially available gamma globulin and its antibodies was used to select proportions between antigen and antibody for the formation of immune precipitates in antigen excess equilibrium and antibody excess Preformed precipitates were washed and mixed with complement in kinetic studies of the inactivation of complement The time for 50 per cent inactivation of complement was recorded Complexes "in statu nascendi" started to inactivate complement with almost no delay while there was regularly a delay before the inactivation produced by preformed immune complexes became demonstrable

Preformed gamma globulin—anti gamma globulin precipitates were heated at 56° C for 60 minutes and the inactivation of complement was compared with the inactivation produced by unheated precipitates

Heating was regularly found to diminish the inactivation effect of immune complexes and this was true both with complexes formed in antibody excess and in antigen excess Controls in which only gamma globulin was mixed with complement and the inactivation followed showed a reverse effect—after heating the gamma globulin produced an increased inactivation of complement

Cederberg A & Gustafsson B E Institute of Histology Lund University HAEMOLYSIN RESPONSE OF GERMFREE RATS TO INTRAPERITONEAL INJECTION OF SHEEP BLOOD CELLS

The response of haemolysin of 8 germfree rats and of a control group of an equal number of normal rats to intraperitoneal injection of sheep blood cells was studied Blood samples for titration were collected at one week intervals for 80 days after the injection The highest values occurred on the 7th day and the maximum titres were substantially higher in the controls than among the germfree animals But the difference of the means was not significant In the control animals however the titres fell rather rapidly in the beginning while the titres for the germfree

animals fell but slightly and through out the rest of the experimental period it was significantly higher (5 6 times) than in the controls

Marked hypergammaglobulinaemia occurred in the control animals An increase in the concentration of the gamma globulins also occurred in the germfree animals *but it was much less marked*

This study will be published elsewhere

Discussion

Fagraeus A The results suggest that the spleen is *responsible for the largest part* of antibody formation in the control animals The antibody curve for the germ free animals suggests that the antibodies are produced in other lymphoid tissues Have any comparative histological investigations been performed on spleen or lymphnodes from the different experimental groups?

Nilsson S B & Skanse B Dep of Clin Chem Malmo allm sjukhus Malmo
EXPERIENCE WITH TESTS FOR ANTIBODIES AGAINST THYROID ANTIGENS

The serological methods for demonstrating antibodies against antigens prepared from *thyroid* are *described* The frequency with which antibodies could be demonstrated in a variety of thyroid diseases in a Britsh series and in a personal series is reported In the present investigation antibodies were demonstrated by the use of the complement fixation reaction and the agglutination of tannin treated red blood cells coated with thyroid antigen

A large hypothyreosis series well defined on the basis of clinical data was studied The results will be published elsewhere

Discussion

Fagraeus A Has delayed hypersensitivity been studied i e cutaneous test with thyreoglobulin?

Norden A Have the authors had the opportunity to follow changes of the antibody titre in individual cases both by the precipitin and complement fixation tests? Are the curves parallel with each other or is perhaps a positive precipitin reaction an expression of a weak auto immune injury to the *thyreoid gland* and a *positive* precipitin plus complement fixation reaction characteristic of widespread injury?

Some fungus infections such as coccidioidomycosis behave in this manner and therefore the serological results allow an assessment of the prognosis

Grubb R It would of course be technically possible to do screen tests for thyroid antibodies in all patients at a department of internal medicine It seems not impossible that it would be as useful as routine Wassermann tests The question requires further study and the usefulness of such a procedure will depend on the frequency with which hypothyreosis is missed and the percentage of non specific reactions in the serological tests

Hallander H & Danielsson D Institute of Bacteriology Uppsala University THE DEVELOPMENT OF ANTIBODIES IN LYMPHOCYTES OF THE THORACIC DUCT

The development of antibodies *in vitro* in the lymphocytes of the thoracic duct was investigated

Cells were taken from the thoracic duct of rabbits immunized with coli 0127

Antibodies were produced in these cells against the bacteria as demonstrated by fluorescent antibodies and agglutination reaction. No cross reactions with 0128 was evident. Washed lymphocytes from immunized rabbits also produced antibodies against diphtheria toxoid in the secondary response but not in the primary response. The antibody titres were measured by the Boyden's haemagglutination technique. Titres was also obtained from lysates of the lymphocytes but the specificity is unclear. The fluorescent antibody technique showed only relatively few cells to be engaged in antibody production. Unna and Giemsa stained preparations showed no obvious plasmacells.

Discussion

Fagraeus A. Tissue culture fluids from spleen cells sometimes give a non specific reaction with tanned cells coated with PPD. We have avoided this complication by adsorbing the culture fluids with tanned cells coated with a different antigen, e.g. bovine gamma globulin.

Juhlin I. & Ericsson C. Dep. of Clinical Bacteriology, Malmo allm. sjukhus, Malmo

A NEW MEDIUM FOR THE BACTERIOLOGIC EXAMINATION OF STOOLS (LSU AGAR)

A new medium (LSU agar) intended for the isolation of *Salmonella* and *Shigella* by inoculation from faeces directly and from enrichment broths was developed. The medium is buffered and contains large amounts of lactose and saccharose, urea and a triple indicator system. It is moderately inhibitory to *Escherichia coli* and *Proteus* whose swarming is completely inhibited. *Salmonella* species including *S. typhi* and *Shigella* grow freely without suppression of their antigen formation. Colonial morphology and colour changes are distinctive. LSU agar proved superior to the Desoxycholate citrate agar and SS agar in promoting the growth of *Salmonella typhi* and *Shigella sonnei*. Parallel routine use of LSU agar and Endo agar [illegible]

[illegible] onset [illegible] antibiotic therapy when the amount of *Salmonella* diminished and *Proteus* increased.

Kjellén L. Dep. of Clinical Bacteriology, Malmo allm. sjukhus, Malmö

A STUDY OF ADENOVIRUS—HOST CELL SYSTEM BY THE PLAQUE TECHNIQUE

Strains of adenovirus type 4 and 5 on cell monolayers form plaques. Plaques can be seen on the 8th day after infection. There is however a continuous increase in number of plaques per plate for at least three to four weeks.

A linear relationship between the concentration of virus seeded and the number of plaques was observed. The frequency distribution of plaques shows agreement with the Poisson distribution.

Approximately 50 per cent of the virus is attached to monolayers in 30–60 minutes.

In a single cycle growth experiment progeny virus was first detected intracellularly at about 18 hours. The peak of virus titre was reached at about 36 hours. The yield obtained amounted to about 10^4 pfu/cell. Newly produced virus remains to a

large extent cell associated Only a few per cent of the progeny virus was found 'free' even 72 hours after infection

Of a thousand cells infected with virus cell ratios of about 80 3 to 8 were capable of forming cell colonies

Paulsson J -F Björklund B & Bjorklund V The State Bacteriological Laboratory Stockholm FURTHER PURIFICATION OF A NORMAL SERUM CYTOLYTIC FACTOR (CF) ACTIVE ON HeLa CELLS *IN VITRO*

Normal serum possess a toxic effect upon atypical cells cultivated in vitro It was succeeded in preparing a cytolytic fraction from a normal serum by means of continuous curtain electrophoresis (*Bjorklund B Proc Soc Exp Biol Med* 103 1 1960 and Fed Proc 19 56 1960) The cytolytic factor (CF) has now undergone further refinement by repeated continuous curtain electrophoresis The technique results in sterile fractions that may be examined by biochemical methods without losing their biological *properties and that may also be observed directly in tissue cultures The cytolytic effect* was *produced* with about one μg and the esterase effect 2-3 μg per ml of the refined fractions These effects and the details of the method are discussed

Discussion

Reichard P A *priori* it seems reasonable to assume that the factor is a protein When one wishes to purify proteins one should always try to combine different methods to utilize separation on different principles In these present case only one parameter was utilized *viz* the migration of the compound during paper electrophoresis This method is actually known to give a very poor separation of proteins which is seen in one of the slides To repeate the same method again does not help If a repeat run is made a different pH should be used

My conclusion must therefore be that the purified factor consists of a mixture of many different substances proteins and compounds of smaller molecular weight

In the authors place I should therefore be extremely hesitant to claim the existence of a special lytic serum factor

Laurell A -B The cells were propagated in medium containing 40 per cent human serum Was some serum also present in the testing system?

Is the serum of the culture medium inactivated or not? Can the lysis of the cells by horse immune serum be interpretated in the conventional way *i e immunolysis* by antibodies and complement?

In Proc Soc Exp Biol Med (1960) Bjorklund writes that CF is dissociated from the inhibitor by dialysis No experimental data confirm this statement On such a dissociation of *CF from the inhibitor CF ought to be activated* How was the material tested after the activating dialysis?

In none of the experiments recorded the cytolysis occurring on addition of immune serum was demonstrated to be caused by the presumed activated CF

Concerning the lytic effect of CF in absence of antibodies the question of preparation artefacts must be considered

Grubb R When the authors claims that so called CF is unique as a factor prepared from human plasma with a direct cytolytic effect I beg to refer to Lthreaus *lysolecithin*

I should be pleased to hear whether the lytic activity of the electrophoretically purified fractions disappears on dialysis

Svedmyr A I would lik, to know if these preparations having a lytic effect on HeLa cells and other establishe l cell lines have been tested in primary cultures of normal human cells Neither in todays lecture nor in Björklunds earlier paper (Proc Soc Exp Biol and Med *1960* 103 1) have such experiments been reported Since it is often seen that HeLa cell cultures are more sensitive to "toxic influences (e g from stool extracts in virus isolation experiments) than are primary cultures of human or monkey cells comparative investigations may be required in wh ch the normal cells are tested with more than the minimal doses active against HeLa cells

Grubb R The phenomena which is referred to as CF has been referred to for about 2 years No basic data with which we might be able to judge the possible biolog cal significance of so-called CF or its supposed importance in cancer has been furnished To be able to form a clearer opinion I would like to ask

1 It is known or have any attempts been made to find out whether so-called CF differs in titre in cancer patients as compared with other patients or with healthy persons?

2 In my opinion the so called immuno activation is an obscure point and it would be interesting to hear *inter alia* which types of antigen antibody reactions give rise to so-called immune activation and which do not

Grubb R Now if the reaction between cellular antigens and their antibodies "causes immuno activation of cytolytic factor one might wonder how it is to be understoood that the s called anti cancer serum does n t produce lysis of normal cells For according to doc Bj rklund himself (Int Arch All & Applied Imm 8 186 1956) normal human cells generally contain at least three cellular antigens aga nst which antib dies are present in the so called anti cancer serum This is true also of such serum to which human plasma has been added in excess

Lun lblad C Pouls ... [illegible]
cal Lal r [illegible]
FRACTIO [illegible]

Normal human serum was split up into 32 fractions with the aid of continuous curtain electrophores s These were tested for protein and ester splitting capacity on substrates such as casein gelatin tosylarginine methyl ester hydrochloride (TAMe) lecithin indoxyl acetate and phenyl acetate These fractions were also tested for the presence of thromb kinase an l prothrombin

A numher of proteolytic activities were dem nstrated s me of them probably never previ usly descril ed

Two aryl esterases were shown One of these was labile and the other stable when treated at 56 C f r 30 minutes One of *them* was totally inactivated by EDTA whereas the presence of EDTA was necessary for the other s activity under the con diti ns of the experiment

Björklun l V Björklun l B & Paulsson J F The State Bacteriological Laboratory Stockh lm AUTOMATIC CONTROL DURING CONTINUOUS CULTURE OF ATYPICAL CELLS OF HUMAN ORIGIN IN "SPINNER" CULTURES

An apparatus f r the c ntinu us culture of atypical cells in Spinner" cultures designed in c llal rati n with Mr T Axelsson and Mr A S Andersson of the Swedish State Bacteri l gical Lab rat ry is descril e l The cell count is kept con

stant by a relay system activated by a photo electric cell which by means of programmating regularly selects the cultures flasks and automatically adds new culture medium and supplies the corresponding amount of cell suspension onto centrifuge flasks The reactive capacity of the control system has been tested with the aid of particles of various kind and found to be within ± 5 per cent The apparatus would appear to have a number of uses within the field of microbiology

Björklund B Bjorklund V & Paulsson J F The State Bacteriological Laboratory Stockholm IMMUNO ACTIVATION OF THE CYTOLYSIS OF ATYPICAL CELLS OF HUMAN ORIGIN (Film)

A lytic system apparently independent of complement has been identified in normal serum (Bjorklund B XIIth Nordic Congr Pathol and Microbiol Gothenburg 1959 Proc Soc Exp Biol Med 103 1 1960, Fed Proc 19 56 1960) The system is present in serum in the form of an inactive complex consisting of a cytolytic factor (CF) bound to an inhibitor (CGI) Both factors have been recovered in relatively pure state from human and equine serum Equine antibodies to antigens from atypical human cells react in vitro in the presence of normal human serum with HeLa cells and other established human cells CF is thus activated which starts fragmentation and lysis of the cells Antibodies alone do not cause cytolysis On the other hand the pure CF free from inhibitor execerised lytic effect even in the absence of antibodies For purposes of demonstration the pure components were added to monolayer cultures of HeLa cells in Rose s chamber whereupon the course of events was elucidated with the aid of time lapse cinephase contrast micrography

Discussion

Laurell A B Bjorklund showed (Int Arch Allergy 1957 *10* 153) *that the cytotoxic* effect of horse immune serum on cancer cells can be eliminated by absorption at +4° C of the antibodies by cancer cells or by purified cell free so called cancer antigen

Such treatment should apparently eliminate the antibodies But should it not also activate CF ? If it does the serum should bring about cytolysis but according to Bjorklund no cytolysis occurs *In 1955 Morgan Mountain prepared antibodies against* HeLa cells by immunization of rabbits She proved the existence of these antibodies by absorption with HeLa cells at +35° C By these procedure her sera like those of Bjorklund lost their cytotoxic effect *But there too the cytolytic factor if such* a factor exists should be activated Has Bjorklund performed the crucial experiment with absorption of the horse antiserum at +35°C ?

The central role of this *CF for the lysis of cancer cells by antibodies must be* questioned until more conclusive experiments have proved its existence

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SUPPLEMENTA